

**REMARKS**

Claims 13, 15 and 24-27 are pending. Claims 1-12, 14, 16-23 and 28-31 are canceled. No claims are herein amended.

In the Advisory Action mailed 2 May 2007, the Examiner has entered Applicants' claim amendments filed 6 April 2007 and has found claims 15 and 24-27 allowable. Applicants express their gratitude. The provisional rejection of claim 13 for obviousness-type double patenting has been maintained over withdrawn claim 6 in co-pending U.S. Appln. Ser. No. 09/854,568. The rejection of claim 13 has also been maintained for obviousness-type double patenting over U.S. Patent Nos. 4,298,590 and 4,486,538, based on inherency, and for anticipation over U.S. Patent No. 4,486,538, also based on inherency.

Claim 13 is directed to a purified monoclonal antibody that specifically recognizes a peptide consisting of the amino acid sequence of SEQ ID NO: 2. SEQ ID NO: 2 is an epitope of the malignin oncoprotein. The Examiner rejects claim 13 because "at least some of the monoclonal antibodies specific to malignin in the prior art have the inherent property of specifically binding to a peptide of SEQ ID NO: 2, as instantly claimed." Advisory Action 2 May 2007 at 2. Applicants respectfully traverse, and request the Examiner withdraw the presently-pending rejection of claim 13.

**I. Interview Summary**

Applicants thank Examiner Emch and Examiner Kemmerer for the fruitful discussion provided during the telephonic Examiner Interview of 18 June 2007. Participants in the Examiner Interview were Examiner Emch and Examiner Kemmerer and Applicants' representatives, Richard Ward of Kenyon & Kenyon LLP and Daren Nicholson of Replikins LLC. The interview summary mailed by the USPTO on June 21, 2007 was received by Applicants; this submission is intended to be responsive to that interview summary.

During the interview, the patentability of claim 13 in the above-captioned application was discussed in view of claim 6 of U.S. Appln. Ser. No. 09/854,568, claims 12-14 of U.S. Patent No. 4,298,590, claims 7-11, 20 and 21 of U.S. Patent No. 4,486,538 and the full disclosure of U.S. Patent No. 4,486,538. Applicants indicated that withdrawn claim 6 in U.S. Appln. Ser. No. 09/854,568 would be canceled, thereby obviating the pending provisional rejection of claim 13 for obviousness-type double patenting. Applicants additionally urged that claim 13 should not be rejected for obviousness-type double patenting over the asserted claims of U.S. Patent Nos. 4,298,590 and 4,486,538, and should not be rejected under 35 U.S.C. § 102(b) over the

disclosure of U.S. Patent No. 4,486,538. Applicants reasoned that the antibody of claim 13 or allegedly-obvious variants of the antibody of claim 13 are not inherently disclosed or claimed in the asserted patents. No claim amendments were discussed and no exhibits were presented.

During the Examiner Interview, Applicants provided and discussed three journal articles in support of their assertion that inoculation of an animal with an antigenic protein likely would not produce antibodies to all epitopes on the antigenic protein: (1) Geysen *et al.*, Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid, PNAS USA, Vol. 81, pp. 3998-4002, July 1984 Biochemistry; (2) Earl *et al.*, Native Oligomeric Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Elicits Diverse Monoclonal Antibody Reactivities, J. Virology, May 1994, p. 3015-3026; and (3) Ditzel *et al.*, Mapping the Protein Surface of Human Immunodeficiency Virus Type 1 gp120 using Human Monoclonal Antibodies from Phage Display Libraries, J. Mol. Biol. (1997) Vol. 267, pp. 684-695. These three articles have been highlighted for the Examiner's convenience, and are attached as Exhibits 1-3 respectively.

Examiner Emch and Examiner Kemmerer requested Applicants submit a Supplemental Response after Final Office Action in support of Applicants' argument that one of skill in the art would understand that antibodies to all epitopes of a protein are not necessarily produced by inoculation of an animal with an antigenic protein. The Examiners further requested Applicants formally submit the three discussed journal articles in a Supplemental Response after Final Office Action.

**II. The Rejection of Claim 13 over Application No. 09/854,568 Is Moot and Applicants Respectfully Request the Rejection Be Withdrawn**

Applicants herewith attach as Exhibit 4, a courtesy copy of a Supplemental Amendment filed 26 June 2007 in co-pending U.S. Appln. Ser. No. 09/854,568. This Supplemental Amendment cancels claim 6 of U.S. Appln. Ser. No. 09/854,568, thereby mooted the provisional double patenting rejection of instant claim 13 made in view of now-canceled claim 6. Withdrawal of this ground of rejection is respectfully requested.

**III. Applicants Respectfully Request the Rejection of Claim 13 Over U.S. Patent Nos. 4,298,590 and 4,486,538 be Withdrawn**

Applicants respectfully submit that the asserted claims of U.S. Patent Nos. 4,298,590 and 4,486,538 do not render claim 13 of the above-captioned application unpatentable for anticipation and/or obviousness-type double patenting. Claim 13, as entered for purposes of appeal, reads:

Claim 13. A purified monoclonal antibody which specifically recognizes a peptide consisting of the amino acid sequence of SEQ ID NO: 2.

In the Advisory Action mailed 2 May 2007, the Office indicated “For purposes of appeal, the proposed amendment(s) . . . will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.” For explanation, the Office, in relevant part, stated:

In addition, the obvious-type double patenting rejections of claim 13 over US Patent No.’s 4,298,590 and 4,486,538 and the rejection of claim 13 under 35 U.S.C. 102(b) as being anticipated by US Patent No. 4,486,538 are maintained for reasons of record. It is irrelevant that the antigenic epitopes were not known, these are inherent to the malignin protien [sic]. Thus, at least some of the monoclonal antibodies specific to malignin in the prior art have the inherent property of specifically binding to a peptide of SEQ ID NO: 2 as instantly claimed.

**A. Applicants submit the “reasons of record” should not apply to claim 13 as now entered**

Claim 13 as originally presented claimed: “A purified monoclonal antibody which specifically recognizes a peptide having the amino acid sequence of SEQ ID NO: 2.” This claim was originally rejected by the Office in an Office Action mailed 22 August 2006. Claim 13, for purposes of appeal, however, claims: “A purified monoclonal antibody which specifically recognizes a peptide consisting of the amino acid sequence of SEQ ID NO: 2.” (emphasis added). The limitation “having the amino acid sequence of SEQ ID NO: 2” is different in scope from the limitation “consisting of the amino acid sequence of SEQ ID NO: 2.” As such, Applicants submit the “reasons of record” should no longer apply. As such, withdrawal of the rejection of claim 13 based on “the reasons of record” is respectfully requested.

**B. To establish inherency, the extrinsic evidence must make clear that the allegedly inherent subject matter is necessarily present, and that it would be recognized by persons of ordinary skill**

Applicants respectfully submit the current rejections of claim 13 in the above-captioned application for obviousness-type double patenting over the asserted claims of U.S. Patent Nos. 4,298,590 and 4,486,538 and for anticipation over U.S. Patent No. 4,486,538 do not have *prima facie* support. One of skill in the art would not expect an antibody that specifically recognizes SEQ ID NO: 2 (such as in claim 13) to be inherently taught by the cited patents because antibodies to all epitopes on an antigenic protein are not necessarily produced by an animal

inoculated with that antigenic protein. Applicants respectfully further submit that the antibody of claim 13 is a species of the genus of anti-malignin antibodies disclosed in the cited patents that is not inherently present in the cited patents.

“The fact that a certain result . . . may occur or be present in the prior art is not sufficient to establish the inherency of that result . . . .” MPEP § 2112(IV) (emphasis in original). “To establish inherency, the extrinsic evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.” *Id.* (emphasis added). “Inherency . . . may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.” *Id.* (emphasis added).

Furthermore, “an invitation to investigate is not an inherent disclosure where a prior art reference discloses no more than a broad genus of potential applications of its discoveries.” *Id.* “A prior art reference that discloses a genus still does not inherently disclose all species within that broad category but must be examined to see if a disclosure of the claimed species has been made or whether the prior art reference merely invites further experimentation to find the species.” *Id.* quoting and explaining *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings*, 370 F.3d 1354, 1367 (Fed. Cir. 2004).

Applicants respectfully submit that one of skill in the art would not expect an antibody that specifically recognizes SEQ ID NO: 2 necessarily to have been produced by inoculation of an animal with the antigenic oncoprotein malignin as described in the cited patents because one of skill in the art would not expect antibodies to all epitopes of the malignin to have been produced in the inoculated animals (absent further manipulation and experimentation that would destroy inherency). Applicants present Geysen *et al.* (Exhibit 1), Earl *et al.* (Exhibit 2) and Ditzel *et al.* (Exhibit 3) in support of the understanding of one of skill in the art and discuss this understanding in Section V below.

**C. The Office has not presented extrinsic evidence that the limitations of claim 13, or allegedly-obvious variants thereof, are “necessarily present” in the cited references**

In the Advisory Action of 2 May 2007, the Office did not identify extrinsic evidence that makes it clear that the recited limitations of claim 13 are necessarily present in claimed embodiments of U.S. Patent Nos. 4,298,590 and 4,486,538, or in allegedly-obvious variants of claimed embodiments. *See* MPEP § 2112(IV). Applicants further submit that such evidence is

also not present in any prior office action. Accordingly, Applicants respectfully request that the rejection of claim 13 be withdrawn.

**D. The Office has not presented evidence a person of ordinary skill would recognize the allegedly-inherent subject matter as necessarily present in the cited art**

The Office has additionally not identified evidence that the limitations recited in claim 13 would be recognized as inherently disclosed in the cited art by a person having ordinary skill in the art. *See id.* Applicants further submit such evidence is also not present in prior office actions.” For this additional reason, Applicants respectfully request that the rejection of claim 13 be withdrawn.

**VI. Claim 13 Should Be Allowed**

It is respectfully submitted that all grounds of rejection of claim 13 have been overcome and that claim 13 should be allowed. Applicants respectfully submit evidence supporting the patentability of claim 13 in Section V below.

**V. Inoculation with Malignin Does Not Provide Inherent Production of an Antibody that Specifically Recognizes SEQ ID NO: 2**

Applicants respectfully submit that one of skill in the art would not expect an antibody that specifically recognizes SEQ ID NO: 2 to have been necessarily produced by inoculation of an animal with the antigenic oncoprotein malignin because one of skill in the art would not expect antibodies to all epitopes of an antigenic protein to be produced by the inoculated animal. Applicants formally submit Geysen *et al.*, Earl *et al.* and Ditzel *et al.* in support of Applicants’ reasoning. These journal articles demonstrate that production of antibodies to all epitopes on an antigenic protein is unlikely when an animal is inoculated with the entire protein. In fact, numerous inoculations and extensive manipulation of a protein is necessary to induce production of antibodies to many epitopes on antigenic proteins.

**A. Geysen *et al.* – antibodies to all epitopes not necessarily produced**

Applicants submit Geysen *et al.* (attached as Exhibit 1) for the proposition that “different animals do not necessarily respond to all of the epitopes on a given antigen.” Geysen at 4001, left column (emphasis added). In Geysen, six different rabbits were inoculated with the whole virus or various particles of the whole virus of Foot and Mouth Disease Virus type O<sub>1</sub>. The inoculations included a whole VP1 protein, the protein on which the investigators hoped to identify all epitopes. Six ELISA scans of the hexapeptides of the 213 amino acid VP1 protein were performed using the six different antisera. *See* Geysen at 3999, right column, Figure 2.

In Figure 2, epitopes on the VP1 protein are illustrated by recording amino acid positions where antibodies from the various antisera bound to the hexapeptides of the protein. A review of Figure 2 reveals that no antisera contained antibodies to all recorded epitopes on the 213 amino acid protein sequence.

In fact, different rabbits produced sera to very different epitopes even when inoculated with the same particle. Scans 1 and 2 illustrate the antibody binding of antisera from two different rabbits inoculated with the same whole virus particle. The authors found the data in Figure 2 demonstrate antibody production in different rabbits inconsistent with regard to the particular epitopes on the protein. The authors wrote:

The two anti-intact virus sera tested, scans 1 and 2, show the extremes in the reactivity patterns found. Large quantitative differences in [] individual animal responses to an identical antigen preparation have been reported before, but these scans highlight the variability possible in the antibody composition between sera.

*Id.* at 3999, right column. Geysen *et al.*, therefore, stands for the proposition that one of skill in the art would not expect individual animals inoculated with the same protein to produce antibodies to all, or even similar, epitopes on the protein. As the authors assert: “[D]ifferent animals do not necessarily respond to all of the epitopes on a given antigen.” Geysen at 4001, left column (emphasis added). As such, one of skill in the art would not expect the anti-malignin antibodies disclosed in the patents cited against claim 13 in the above-captioned application to produce antibody that specifically recognizes the epitope of SEQ ID NO: 2, as required by claim 13.

**B. Earl *et al.* – antibodies to linear epitope like SEQ ID NO:2 not necessarily produced**

Applicants submit Earl *et al.* (attached as Exhibit 2) for the proposition that linear epitopes, such as SEQ ID NO: 2 of malignin, are not necessarily recognized by antibodies induced to an antigenic protein like malignin. In Earl, a very large number of monoclonal antibodies were raised to both the quaternary and monomeric structure of HIV-1 envelope glycoprotein. The monoclonal antibodies exhibited extensive variability in affinity for particular epitopes, especially linear epitopes. For example, in monoclonal antibodies raised in seven different animals against the quaternary structure of the protein, less than 7% of the antibodies recognized linear epitopes in the important V3 loop of the protein. Earl at 3015, Abstract. Further, in monoclonal antibodies raised against the monomeric protein, less than half of the antibodies recognized linear epitopes in the V3 loop. *Id.* The authors found that monoclonal

antibodies were more likely to recognize conformational epitopes than linear epitopes. *Id.* at 3016, left column (noting linear epitopes in the C-terminal portion of gp120 were not obtained and relatively few antibodies were obtained against linear epitopes in the V3 loop). As such, one of skill in the art would not expect inoculation of an animal with antigenic malignin to necessarily result in production of an antibody that specifically recognizes the linear epitope of SEQ ID NO: 2.

**C. Ditzel *et al.* – producing antibodies to range of epitopes requires extensive selection strategies**

Applicants submit Ditzel *et al.* (attached as Exhibit 3) for the proposition that producing antibodies to a range of epitopes on a protein requires “a huge body of work in the generation of antibodies alone” and “a number of different selection strategies.” Ditzel at 685, left and right columns. In Ditzel, mapping epitopes on the protein surface of HIV-1 gp120 with a phage display library “required the input of antibodies from many laboratories and represent[ed] a huge body of work in the generation of the antibodies alone.” *Id.* at 685, left column. The mapping process required generating antibodies to quaternary and monomeric versions of the protein, to individually- and multiply-masked proteins, to linear peptides corresponding to small segments of the protein and to a constrained peptide corresponding to the crown of the important V3 loop. *Id.* at 685, left column. In one example, a 24 amino acid residue of the V3 loop was used to select for an antibody to the V3 loop. *Id.*

The authors concluded that to “obtain antibodies to a range of epitopes[,] may require more than simple selection of the library against the antigen of interest.” *Id.* at 691, left column. Instead, the authors described a set of “selection procedures leading to the isolation of an extended set of specificities to a single antigen.” *Id.* As such, one of skill in the art would not understand the disclosure of U.S. Patent Nos. 4,298,590 and 4,486,538 to necessarily present an antibody that specifically recognizes SEQ ID NO: 2. MPEP § 2112(IV). In fact, one of skill in the art would understand that the cited references disclose “a broad genus of potential applications” of anti-malignin antibody that “does not disclose[] the claimed species” of claim 13 and, at best, “merely invites further experimentation to find the [claimed] species.” *Id.* One of skill in the art would further understand that such experimentation to find the claimed species would require “more than simple selection” of antibodies using a phage display library and perhaps “a huge body of work in the generation of antibodies alone” along with “a number of different selection strategies.” Ditzel at 685, left column. As a result, one of skill in the art would recognize that the antibody of claim 13 in the above-captioned application is not inherently disclosed in U.S. Patent No. 4,298,590 or in U.S. Patent No. 4,486,538.

**CONCLUSION**

It is believed that the present claims are in condition for allowance and Applicants earnestly request the same. It is not believed that an extension of time is required for this submission; however, if the USPTO disagrees, extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a) and any fees required therefor are hereby authorized to be charged to Kenyon & Kenyon LLP Deposit Account No. 11-0600.

The Examiner is invited to contact the undersigned attorney if necessary to expedite allowance. An early and favorable action on the merits is earnestly solicited.

Respectfully submitted,

KENYON & KENYON LLP

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# EXHIBIT 1

## Use of Peptide Synthesis to Probe Viral Antigens for Epitopes to a Resolution of a Single Amino Acid

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*PNAS* 1984;81;3998-4002  
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Notes:

# Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid

(antigenic determinant/foot-and-mouth disease virus)

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Communicated by G. J. V. Nossal, March 12, 1984

**ABSTRACT** A procedure is described for rapid concurrent synthesis on solid supports of hundreds of peptides, of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner an immunogenic epitope of the immunologically important coat protein of foot-and-mouth disease virus (type O<sub>1</sub>) is located with a resolution of seven amino acids, corresponding to amino acids 146–152 of that protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope was synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. It was found that the leucine residues at positions 148 and 151 were essential for reaction with antisera raised against intact virus. A lesser contribution was derived from the glutamine and alanine residues at positions 149 and 152, respectively. Aside from the practical significance for locating and examining epitopes at high resolution, these findings may lead to better understanding of the basis of antigen–antibody interaction and antibody specificity.

Recombinant DNA technology now makes possible by deduction from the determined nucleotide sequences reliable amino acid sequences of biologically important proteins. However, methods for identifying the loci in a protein that constitute the antigenic and immunogenic epitopes are few and time consuming and form the bottleneck to further rapid progress. Immunogenic epitopes are defined as those parts of a protein that elicit the antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule (1–3). On the other hand, a region of a protein molecule to which an antibody can bind is defined as an antigenic epitope. Antisera prepared against chemically synthesized peptides corresponding to short linear tracts of the total polypeptide sequence have been shown to react well with the native protein (4–9). However, interactions were also found to occur even when the site of interaction did not correspond to an immunogenic epitope of the native protein. This has been interpreted to mean that the number of immunogenic epitopes of a protein is less than the number of antigenic epitopes (4). Conversely, since antibodies produced against the native protein are, by definition, directed to the immunogenic epitopes, it follows that peptides reacting with these antibodies must contain elements of the epitopes. From a study of the few proteins for which the determinants have been accurately mapped, it is postulated that a determinant may consist of a single element (continuous) or of more than one element brought together from linearly distant regions of the polypeptide chain by the folding of that chain as it exists in the

native state (discontinuous) (10). Systematic mapping of all the detectable reactive elements of a protein by the chemical synthesis of overlapping segments has until now been severely limited by the scale of the synthetic and testing capability required (10, 11). Smith and co-workers (12, 13) circumvented the decoupling and purification steps by combining solid-phase peptide synthesis and solid-phase radioimmunoassay using the same solid support.

We describe here the concurrent synthesis of all 208 possible overlapping hexapeptides covering the total 213-amino acid sequence of the immunologically important coat protein (VP1) of foot-and-mouth disease virus (FMDV), type O<sub>1</sub> (Fig. 1). The peptides, still attached to the support used for their synthesis, were tested for antigenicity by an ELISA using a variety of antisera. After identification of a hexapeptide reactive with antibody raised against the intact virus, all 120 hexapeptides representing the complete single point amino acid replacement set were synthesized and tested for retention of antigenicity. By this method a whole virus epitope was examined at a resolution of a single amino acid.

## MATERIALS AND METHODS

**Synthesis of Peptides.** Polyethylene rods (diameter, 4 mm; length, 40 mm) immersed in a 6% (vol/vol) aqueous solution of acrylic acid were  $\gamma$  irradiated at a dose of 1,000,000 rads (1 rad = 0.01 gray) (15). Rods so prepared were assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in a Teflon tray with a matrix of wells to match the rod spacing. Conventional methods of solid-phase peptide chemistry (16, 17) were used to couple *N*<sup>ε</sup>-*t*-butyloxycarbonyl-L-lysine methyl ester to the polyethylene/polyacrylic acid via the *N*<sup>ε</sup>-amino group of the side chain. Carboxy substitution of the support was determined by treating NH<sub>2</sub>-lysine(OMe)-polyethylene/polyacrylic acid with <sup>14</sup>C-labeled butyric acid and was found to be 0.15–0.2 nmol/mm<sup>2</sup>. Removal of the *t*-butyloxycarbonyl group was followed by the coupling of *t*-butyloxycarbonyl-L-alanine to complete a peptide-like spacer. Successive amino acids were added as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the *t*-butyloxycarbonyl protecting group, the terminal amino group was acetylated with acetic anhydride in dimethylformamide/triethylamine. All *N,N*-dicyclohexylcarbodiimide-mediated coupling reactions were carried out in dimethylformamide in the presence of *N*-hydroxybenzotriazole. The following side-chain protecting groups were used: *O*-benzyl for threonine, serine, aspartic acid, glutamic acid, and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methylbenzyl for cysteine; and 1-benzyloxycarbonylamido-2,2,2-trifluoroethyl for histidine. Side-chain-protecting groups were removed by treatment with borontris(trifluor-

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Abbreviations: FMDV, foot-and-mouth disease virus; P<sub>i</sub>/NaCl, phosphate-buffered saline.

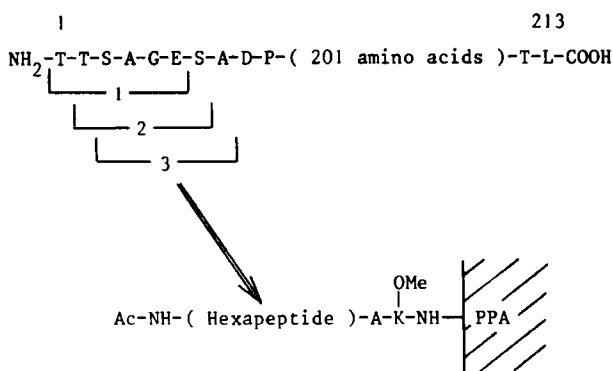


FIG. 1. The 213-amino acid sequence of VP1 (FMDV, type O<sub>1</sub>) as translated by Kurz *et al.* (14) was subdivided into hexapeptide units, and each was synthesized on a separate polyethylene support in the orientation, and with a dipeptide spacer, as shown. Peptides are numbered according to the position of the NH<sub>2</sub>-terminal amino acid within the VP1 sequence. PPA, polyethylene/polyacrylic acid.

acetate) in trifluoroacetic acid for 90 min at room temperature (18). After hydrolysis with HCl/propionic acid, sequences included in the synthesis as controls were analyzed to confirm that, although coupling at each stage had occurred, it was incomplete for several of the amino acids, notably arginine. Before testing by ELISA, support-coupled peptides were washed several times with phosphate-buffered saline (P<sub>i</sub>/NaCl).

**Antisera.** Antisera against the intact virus particle were prepared by immunizing rabbits with 50  $\mu$ g of inactivated, density gradient-purified virus in complete Freund's adjuvant. The animals were bled 3–4 weeks after the single inoculation. Anti-virus-subunit serum was prepared by inoculating rabbits three times, 3–4 weeks apart, with 10  $\mu$ g of acid-disrupted purified virus, initially in complete Freund's and subsequently in incomplete Freund's adjuvant. The polypeptide VP1 was separated from the mixture of proteins obtained from urea-disrupted purified virus by isoelectric focusing (19). It was eluted from the gel with 8 M urea and dialyzed against P<sub>i</sub>/NaCl, and antiserum was raised in rabbits as described for the virus subunit. Antiserum for scan 3 (see Fig. 2) was that used for scan 2 after absorption with purified virus (1500  $\mu$ g of whole virus was incubated with 1 ml of serum for 72 hr at 4°C), and all virus-bound antibodies were removed by centrifugation.

**ELISA.** Support-coupled peptides were precoated with 10% horse serum/10% ovalbumin/1% Tween 80 in P<sub>i</sub>/NaCl for 1 hr at 37°C to block nonspecific absorption of antibodies. Overnight incubation at 4°C in a 1:40 dilution of antiserum in the preincubation mixture was followed by three washes in 0.05% Tween 80/P<sub>i</sub>/NaCl. Reaction for 1 hr at 37°C with a 1:50,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase in the preincubation mixture was again followed by extensive washing with P<sub>i</sub>/NaCl/Tween to remove excess conjugate. The presence of antibody was detected by reaction for 45 min with a freshly prepared developing solution (40 mg of *o*-phenylenediamine and 20  $\mu$ l of hydrogen peroxide in 100 ml of phosphate buffer, pH 5.0), and the color produced was read in a Titertek Multiskan (Flow Laboratories, Melbourne, Australia) at 420 nm. Prior to retesting, bound antibody was removed from the peptides by washing peptides three times at 37°C in 8 M urea/0.1% 2-mercaptoethanol/0.1% sodium dodecyl sulfate and then several times with P<sub>i</sub>/NaCl.

## RESULTS

**Identification of a Virus Particle-Associated Immunogenic Epitope.** All 208 possible hexapeptides from the amino acid

sequence of the VP1 protein of FMDV type O<sub>1</sub> were synthesized in duplicate. The amino acid sequence had been deduced from the nucleotide sequence of the VP1 gene (14). The results obtained for all the synthesized hexapeptides when tested by ELISA with six different antisera are shown in Fig. 2. Antisera used in the test were as follows: two different anti-(intact virus, type O<sub>1</sub>), a virus-absorbed anti-(intact virus, type O<sub>1</sub>), an anti-(virus subunit, type O<sub>1</sub>), an anti-(isolated virus protein VP1, type O<sub>1</sub>), and, as a control, an anti-(intact virus, type C<sub>3</sub>). The two anti-intact virus sera tested, scans 1 and 2, show the extremes in the reactivity patterns found. Large quantitative differences in the individual animal responses to an identical antigen preparation have been reported before, but these scans highlight the variability possible in the antibody composition between sera. Examination of scans 1, 2, and 3 shows that antibodies reactive with hexapeptide numbers 146 and 147 are present in anti-intact particle sera (scans 1 and 2) but completely absent after absorption of the sera with purified virus (scan 3). Presumably, scan 3 registers those antibodies raised against epitopes expressed in denatured virions that are not present on the surface of the intact virion. Activities to hexapeptides 146 and 147 were not observed in the anti-subunit serum (scan 4) and were only weakly present in the anti-VP1 serum (scan 5). That some activity was found in the anti-VP1 serum

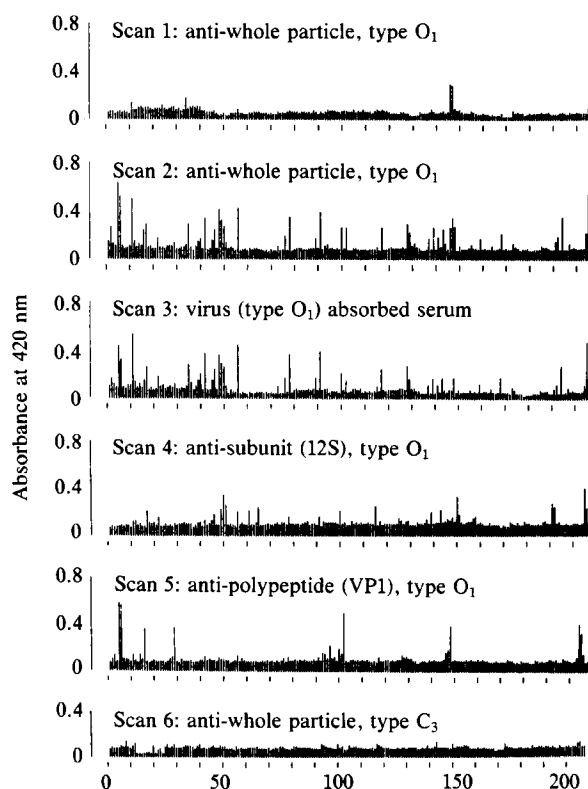


FIG. 2. Antigenic profiles (scans). Results are shown as vertical lines proportional to the extinction obtained in the antibody-binding ELISA test, plotted above the number giving the location within the VP1 sequence of the NH<sub>2</sub>-terminal amino acid of each peptide. Antisera used to produce the scans shown were as follows: 1 and 2, two different anti-whole virus particle, type O<sub>1</sub>; 3, anti-whole virus particle (as used in 2) after absorption with purified intact virus; 4, anti-virus subunit, type O<sub>1</sub>; 5, anti-VP1, type O<sub>1</sub>; 6, anti-whole virus particle, type C<sub>3</sub>. It should be noted that, because the sequence of VP1 contains 20 alanine residues, 20 of the peptides synthesized match for seven amino acids. However, the frequency of reactive peptides from this group was not significantly different from the overall frequency (0.2 compared with 0.16) and therefore not considered further.

possibly accounts for the immunizing capacity, albeit weak, of the isolated protein (20). It should be noted however that another anti-VP1 serum tested, while retaining a strong activity at position 148, showed no activity at positions 146 and 147. Comparison of scan 3 with scan 2 (absorbed compared with nonabsorbed) shows that, in addition to the loss of activity to peptides 146 and 147, some reduction in activity to peptides 5, 6, and 206 also occurred. Of these, activity to 5 and 6 was not found in all the anti-intact virus sera tested, but activity to 206 was invariably present. From this we conclude that of the peptides found to be reactive, the pair at 146 (G-D-L-Q-V-L) and 147 (D-L-Q-V-L-A) [in this paper, amino acids are identified by the single-letter code (21)] constitute or are part of the principal immunogenic epitope, with the element at 206 (V-A-P-V-K-P) contributing to a lesser epitope. This is consistent with the observations of others (5, 22). Scan 6 shows the absence of reactivity in an antiserum produced against a different serotype of the virus.

**Extending the Resolution of the Epitope at Peptides 146/147 to a Single Amino Acid.** From the preceding data, we were unable to distinguish between two possibilities: (i) the epitope is contained in the five amino acids common to peptides 146 and 147—i.e., D-L-Q-V-L—or (ii) the epitope is represented by the “sum” of the two hexapeptides—i.e., G-D-L-Q-V-L-A. To extend the resolution, all 120 possible hexapeptides differing from peptide 146 (G-D-L-Q-V-L) by only a single amino acid were synthesized. Each of the other 19 common amino acids was substituted in each of the six amino acid positions within the peptide. Positions at which all or at least the majority of substitutions result in a loss of antibody-binding activity indicate those residues that are important for the specificity and binding to antibody. The ELISA activity obtained for each of the 120 peptides when serum 48 (anti-intact virus particle) was used in the test are shown in Fig. 3. The relative activities (with respect to the parent sequence) determined for each peptide for two different anti-intact virus sera, nos. 31 and 48, are given in Table 1. To determine the contribution of the alanine residue (carboxyl terminus of peptide 147) toward reactivity and/or specificity, a further 20 peptides were synthesized. Each of these peptides consisted of the complete sequence of 146 (G-D-L-Q-V-L) with one of the 19 possible amino acids added to the carboxyl terminus and synthesized as described before. When serum 31 was used in the test, activity was retained for seven of the amino acids. Relative values expressed in the same way as given in Table 1 were as follows: A (parent amino acid), 99; D, 55; E, 36; G, 45; N, 95; Q, 98; S, 44. With

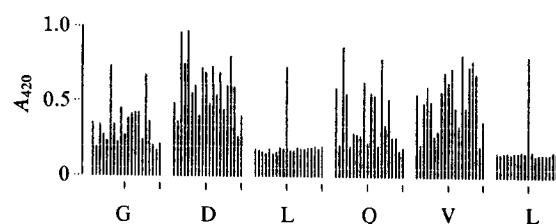


FIG. 3. Antibody-binding activity. The result for each peptide is shown as a vertical line proportional to the ELISA extinction obtained. Every group of 20 lines corresponds to the complete replacement set for one of the six amino acid positions in the hexapeptide G-D-L-Q-V-L. Within each group of 20 lines, the left-hand line corresponds to the substitution of the original residue by alanine (A), and the successive lines are then in alphabetic order according to the single letter code for the amino acids.

serum 48, activity was retained for four amino acids: A (parent amino acid), 94; G, 30; S, 47; T, 39.

## DISCUSSION

**Interpretation of Data.** In choosing to adopt the procedure for peptide synthesis as described, we made several assumptions.

1. To detect antibodies, the quantity of peptide of a defined sequence need only be in the pmol range (5). Assuming a worst-case overall yield of 1% for an eight-step synthesis (two linking and six sequence amino acids), an initial level of 1 nmol of reactive group per support would satisfy the above condition.
2. High purity for the peptide used in the detection of antibodies is not a necessary condition. The majority of serological tests rely on the specificity of antibodies to detect a given antigen in the presence of large amounts of irrelevant protein.
3. Except for cases in which either all or none of the peptides react, a large number of the peptides would effectively act as negative controls in the test. With adjacent peptides sharing a common sequence of five amino acids, the observation of peaks above a generally uniform background level would indicate a valid test.
4. Many of the antibodies elicited by immunization with an intact virus result from presentation of epitopes in fully or partially denatured form. Such antibodies may bind to synthetic peptides *in vitro* but not to the virion itself. They are therefore assumed to be less relevant to virus neutralization

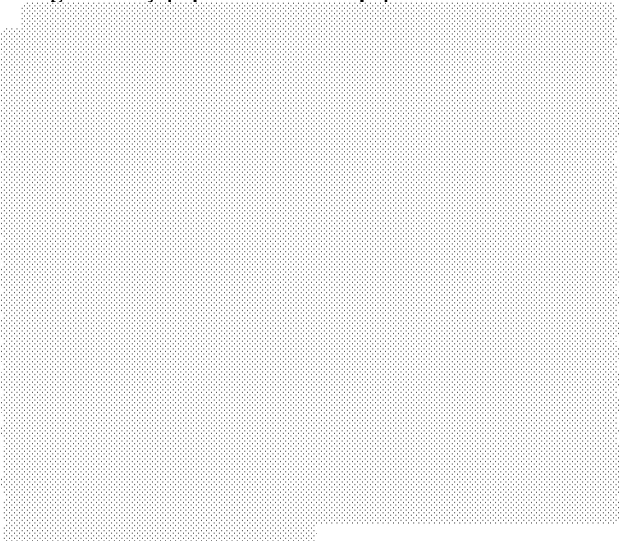
Table 1. Relative antibody-binding activities of peptides derived from the parent sequence G-D-L-Q-V-L

Serum	Parent residue	Activity when substituted with amino acid																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
31	G	29					<b>90</b>	14	27	12		32	34	41	29		50				
	D	22	21	<b>143</b>	95	110	28	65		12	65	15	58	10	69		38	62			
	L										<b>79</b>										
	Q				64	14							13		<b>80</b>						
	V	62		33	52				26		29	59			45		49	43	<b>89</b>		
	L										<b>119</b>										
48	G	11					<b>88</b>	10		32		18	24	25	26		77	14			
	D	37	12	<b>136</b>	92	137	52	62	21	87	81	37	89	49	80	29	63	104	60		21
	L										<b>88</b>										
	Q	60		117	52					68		53	49		<b>102</b>	10	45				
	V	52		40	63	42			56	82	68	88	34		106	33	91	98	<b>81</b>		14
	L										<b>105</b>										

Antibody-binding activities are shown for all peptides that gave an extinction significantly above background. Values for each peptide are expressed as a percentage of the mean activity of the six parent sequences synthesized as a part of each replacement set. Values given boldface type correspond to those obtained for the parent sequence. No activity was detected when the antiserum used was prepared against the heterologous FMDV type.

than are antibodies that bind to virions as well as to peptides.

The extinction obtained in an ELISA for a given peptide depends on the concentration of the antibody population with the correct specificity for reaction. It is essentially independent of the peptide density expressed as reacting groups per mm<sup>2</sup> of support (unpublished data). The difference in the extinction obtained with peptides synthesized with densities varying over two orders of magnitude is similar to the 10–30% variation observed between replicate synthesis (unpublished data). The extinction may also be expected to depend greatly on the affinity between peptide and reacting antibody, but this remains to be verified, although the overnight reaction would tend to minimize differences. Antigenic profiles of the FMDV VP1 (Fig. 2) were interpreted to define an antigenic peptide as one giving an ELISA extinction significantly above the background level of the test. On the other hand, in the testing of replacement nets (Fig. 3), the concentration of the reactive antibody population is constant and effectively of one specificity. Therefore, the extinctions observed are interpreted to reflect the mean affinity of the reacting antibody population for the peptide.



**An Immunogenic Epitope at High Resolution.** Antibodies raised against a particular immunogenic epitope will have a combining site (paratope) complementary to the structure of that epitope. An antibody population directed to the same epitope (allowing for variation in the expression of antibodies by the immune response) will have common features in the combining sites essential for binding to that epitope. A peptide that, in one of its many conformations in thermal equilibrium *in vitro*, has a structure sufficiently similar to the form of the epitope against which antibody was raised *in vivo* will bind to the antibody. Modification of a reacting peptide by amino acid substitution will define the limits for interaction with antibody. By so “mapping” the antibody-combining site, it is possible to infer properties of the antigen to which this antibody population is complementary. Using polyclonal antisera, it was not expected that a rigorous requirement for particular amino acids in particular positions would be observed. It is clear that, whatever the diversity of the antibodies involved in the interaction, the requirement for a given amino acid in certain positions is absolute for most or all of the antibodies present. It is also clear that the specificity range found for the two different antisera is remarkably similar, differing mainly in the hierarchy of preference for amino acids at the nonessential position. As judged from the limitation to replacements at some position within the sequence G-D-L-Q-V-L-A, the whole-virus epitope may be considered to be X-X-L-Q-X-L-A, where X is nonessential, letters in boldface type indicate an absolute require-

ment, and letters in lightface type indicate a contributing amino acid.

These findings suggest a different interpretation of the characteristics of epitopes. The antigenic specificity of the epitope represented by amino acids 146–152 within the VP1 protein of FMDV is largely dependent on the leucine residues at positions 148 and 151. These are hydrophobic residues and would not normally be expected to protrude from the protein surface. This suggests the possibility that the immune system responds to a local protein conformation that is different from that expected to represent the global energy minimum. The energy for antigen–antibody binding may be derived from the positive entropy term associated with the transfer of hydrophobic residues from a hydrophilic (aqueous) environment to within the antibody-combining site.

**Scope of the Described Approach to Epitope Mapping.** Although our results have been presented for a single protein only, the agreement with results of others in locating a viral epitope within the region encompassing amino acids 141–160 of VP1 is excellent (5, 22). The further resolution obtained by Rowlands *et al.* (25) from the comparison of the sequences of the VP1s of three antigenic variants of a single virus type (A<sub>12</sub>) showed that amino acid substitution at positions 148 and/or 153 would affect the ability to react with specific antibody. This result is in good agreement with our results for subtype O<sub>1</sub>, where positions 148 and 151 were critical to the immunogenicity of the epitope. We expect that the systematic approach as outlined, when applied to a broader spectrum of proteins, will contribute greatly to our understanding of the nature of epitopes and their interaction with the immune system.

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## EXHIBIT 2

# Native Oligomeric Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Elicits Diverse Monoclonal Antibody Reactivities

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**We synthesized and purified a recombinant human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein, lacking the gp120/gp41 cleavage site as well as the transmembrane domain, that is secreted principally as a stable oligomer. Mice were immunized with separated monomeric and oligomeric HIV-1 Env glycoproteins to analyze the repertoire of antibody responses to the tertiary and quaternary structure of the protein.**

The human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein is a structurally and functionally complex integral membrane protein that plays a number of important roles in virus infection. In addition, it is a target of humoral and cell-mediated immune responses (48, 57, 71). Env is synthesized as a polyprotein precursor, gp160, which is cleaved during transport into gp120 and gp41 subunits (1, 80, 83). The external gp120 subunit is noncovalently associated with gp41 and is responsible for binding virus to CD4 molecules which serve as the cellular receptor (12, 42). The gp41 subunit is anchored in the membrane by virtue of a hydrophobic transmembrane domain (23, 29). Following specific receptor binding, the Env glycoprotein presumably undergoes a conformational change and initiates fusion between the viral envelope and the target cell through the action of the fusion domain of the gp41 molecule (4, 44).

Prior to cleavage, gp160 molecules assemble into noncovalently associated dimers and a higher-order structure, most likely consisting of a dimer of dimers (14, 66, 73). The ectodomain of gp41 is largely responsible for subunit assembly (14, 19, 78). Oligomerization of the Env glycoprotein has several consequences. First, it is required for transport from

the endoplasmic reticulum; mutants that fail to oligomerize are retained and degraded (84). Second, multimeric CD4 binding by Env oligomers may facilitate viral entry (15, 49, 54). Third, as for some other viral proteins (82), Env quaternary structure may have important antigenic implications (6, 66, 68, 76).

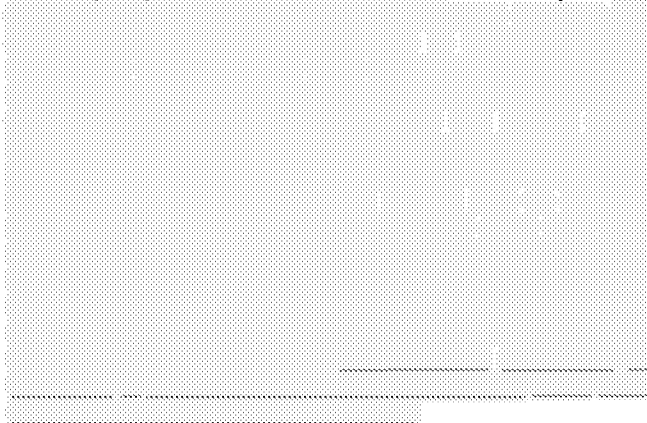
There are several reasons to suspect that the oligomeric nature of Env may influence its antigenic structure. Multimeric proteins typically interact over large areas, making structural differences between oligomeric and monomeric subunits likely (39). In the case of HIV-1 Env, nearly the entire gp41 ectodomain is required for stable subunit association (19). Consequently, it is not surprising that some antibodies react more strongly with oligomeric than monomeric gp41 (6, 66, 68). There is also evidence for contacts between adjoining gp120 subunits (19, 62, 81). Antibodies to conformation-dependent epitopes in gp120 are prevalent in the sera of infected individuals and account for the majority of the neutralizing activity (35, 53, 76). In some instances, a significant amount of this neutralizing activity cannot be accounted for by antibodies directed against epitopes in monomeric gp120 (76). Thus, epitopes contingent upon Env quaternary structure to which neutralizing antibodies are directed may exist not only in gp41 but in gp120 as well.

In this report, we describe the production and purification of secreted oligomeric HIV-1 Env protein. Mice were immunized with monomeric, dimeric, or tetrameric HIV-1 Env glycoprotein isolated under nondenaturing conditions to study the effects of Env quaternary structure on the repertoire of antibodies elicited by each form of the protein. Furthermore,

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screening of monoclonal antibodies (MAbs) was performed by immunoprecipitation with the same undenatured Env proteins.



## MATERIALS AND METHODS

**Recombinant vaccinia viruses.** For production of soluble, secreted HIV-1 Env glycoprotein, two recombinant vaccinia viruses were constructed. The *env* gene from the BH8 isolate (GenBank accession number KO2011) was used (55, 70), and nucleotide numbers are derived from this sequence. Numbering of amino acids begins at the start of the open reading frame and thus includes the signal peptide. For construction of both recombinant viruses, two translation termination codons were inserted after the lysine residue at amino acid 678 (nucleotide 2034), just prior to the transmembrane domain of gp41, using a two-step PCR protocol (38). In the first step, two fragments with overlapping ends were synthesized. These spanned a region of the *env* gene from the *Hind*III restriction site (nucleotide 2128), through the transmembrane coding region, to the *Bam*HI restriction site (nucleotide 2462). One fragment was generated with the synthetic oligonucleotide a (5'-AA CAATTACACAAGCTTAATACACTC-3'), containing the *Hind*III restriction site, in conjunction with oligonucleotide b (5'-CCCCGCGGTTATTATTTATATACCACAGCCA ATTTGT-3'), containing the translation termination codons. The other fragment was generated with oligonucleotide c (5'-GTGCTAAGGATCCGTTCACTAATCG-3'), containing the *Bam*HI restriction site, in conjunction with oligonucleotide d (5'-TAATAACCGCGGGGTTATTCATAATGATAGTA GGAGGC-3'). These two fragments were then used together in a second reaction along with oligonucleotides a and c to generate a 372-bp fragment. This fragment was digested with *Hind*III and *Bam*HI and exchanged with the analogous fragment in the *env* gene of pSC60 (7), a plasmid which contains the entire HIV-1 *env* gene under control of a synthetic early/late vaccinia virus promoter. The resulting plasmid, pCB-14, thus contains the *env* gene truncated after amino acid 678. The proteolytic cleavage sites between gp120 and gp41 were removed by substitution of a 575-bp *Ssp*I-*Hind*III fragment between nucleotides 1553 and 2128 with the analogous fragment from pPE12 (17) to generate pPE12B. Plasmid pPE12 contains the *env* gene from which 12 amino acids including the primary and secondary cleavage sites have been removed. Plasmids pCB-14 and pPE12B were used to generate recombinant vaccinia viruses vCB-14 and vPE12B (18), which express cleavable and noncleavable secreted gp140 molecules, respectively.

Several other recombinant vaccinia viruses were also used. vPE16 (16) and vSC60 (7) express wild-type gp160 under the vaccinia virus 7.5K and synthetic early/late promoters, respec-

tively; vPE12 expresses a noncleavable form of gp160; vPE8 expresses gp120; and the series vPE17, vPE18, vPE20, vPE21, and vPE22 (17) express C-terminally truncated Env molecules. vSC64 expresses a chimeric Env glycoprotein molecule consisting of HIV-2 gp120 and HIV-1 gp41 (7). vCB-5 (5) expresses soluble CD4 (sCD4) (372 amino acid residues). In addition, plasmid pPE63 (19), expressing truncated Env glycoprotein via the hybrid vaccinia virus-T7 system (21), was used.

**Purification of soluble, secreted HIV-1 Env glycoprotein gp140 for immunizations.** Typically, 40 confluent 160-cm<sup>2</sup> flasks, each containing approximately  $1.5 \times 10^7$  BS-C-1 cells, were infected with vPE12B at a multiplicity of infection of 10. At 2 h after infection, the monolayers were washed three times with phosphate-buffered saline (PBS) to remove free virus particles and then overlaid with serum-free OPTI-MEM (GIBCO, Grand Island, N.Y.). After 24 to 36 h, the medium was harvested and culture debris was removed by centrifugation for 30 min at 12,000 rpm. Triton X-100 was then added to 0.5% (vol/vol) final concentration and maintained through lentil lectin chromatography in order to prevent nonspecific binding of proteins with the column material. Glycoproteins were purified by lentil lectin-Sepharose (Pharmacia, Piscataway, N.J.) chromatography as follows. The pooled culture supernatant containing secreted gp140 was cycled continuously over a column (1 by 13 cm) overnight. The column was washed with PBS containing 10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, and 0.5% Triton X-100 (10 column volumes) followed by PBS containing 10 mM Tris-HCl (pH 8.0) (2 column volumes). Glycoproteins were eluted with 0.5 M methyl  $\alpha$ -D-mannopyranoside in PBS containing 10 mM Tris-HCl (pH 8.0) (3 column volumes) and concentrated 20- to 30-fold in Centricon microconcentrators (Amicon). This material was loaded onto 5 to 20% sucrose gradients in the absence of detergent and centrifuged in an SW40 rotor for 20 h at 4°C at 40,000 rpm (14). After fractionation, a small aliquot of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (immunoblotting) using a rabbit polyclonal antiserum to HIV-1 gp160 (R160) (84) and <sup>125</sup>I-labeled protein A (Amersham). Fractions containing monomeric, dimeric, and tetrameric Env glycoprotein were pooled and concentrated. To verify the oligomeric status of each pooled fraction, aliquots were cross-linked with 1 mM ethyleneglycol bis(succinimidylsuccinate) (EGS; Pierce, Rockford, Ill.) and analyzed by SDS-PAGE (4% gel) and Western blotting with R160 as described previously (14). Purified Env glycoprotein preparations were separated by SDS-PAGE (10% gel) and visualized with Coomassie blue G250.

**Immunization of mice and production of hybridomas.** A.SW/SnJ mice (Jackson Laboratory, Bar Harbor, Maine) were immunized with either monomeric, dimeric, or tetrameric Env glycoprotein preparations with RiBi adjuvant (RiBi Immunochem Research Inc., Hamilton, Mont.) as recommended by the supplier. Briefly, mice were inoculated at 3-week intervals with 15 to 20  $\mu$ g of purified HIV-1 Env glycoprotein per mouse (one-half subcutaneously and one-half intraperitoneally). A test bleed was performed following the first boost, and the sera were assayed by both immunoprecipitating and immunoblotting to ensure reactivity with the immunogen. Mice receiving monomeric and dimeric Env preparations were inoculated three times, while mice receiving tetrameric Env were inoculated four times.

Three days after the final inoculation, mice were sacrificed and the spleens were harvested and prepared for cell fusion by standard methods (22). Splenocytes were fused with Sp2/0 myeloma cells (ATCC 1581) with polyethylene glycol, using a

modification of the method of Gefter et al. (24). Following polyethylene glycol fusion, the cell preparations were distributed in 96-well plates at a density of  $10^5$  cells per well, based on the number of Sp2/0 partner cells, and selected in Iscove's minimal essential medium supplemented with hypoxanthine-aminopterin-thymidine, 10% fetal calf serum (HyClone Laboratories, Hazelton, Mont.), and 100 U of recombinant interleukin-6 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. No feeder cell cultures were used. The medium was replaced with fresh hypoxanthine-aminopterin-thymidine medium approximately 10 days after plating. Twelve days after plating, the supernatants from all wells containing a hybridoma colony were screened by immunoprecipitation with iodinated Env glycoprotein preparations. Spleens from the two mice immunized with monomeric Env yielded 378 hybridoma colonies, of which 15 were reactive with Env by immunoprecipitation. Spleens from the three mice immunized with dimeric Env and from the four mice immunized with tetrameric Env yielded 733 and 1,044 hybridoma colonies and 68 and 55 anti-Env MAbs, respectively.

**Radiolodination of HIV-1 Env glycoproteins.** Preparations (50  $\mu$ g) of purified monomeric, dimeric, or tetrameric HIV-1 Env glycoproteins were labeled with  $^{125}$ I by the chloramine T method with Iodobeads (Pierce Chemical) as described by Markwell (51). Radiolabeled glycoproteins were separated from free iodine by passage over a Sepharose G-25 column and stored at 4°C. Typically, the specific radioactivity of Env glycoprotein was approximately  $1 \times 10^6$  to  $2 \times 10^6$  cpm/ $\mu$ g. Iodination of Env glycoprotein preparations did not disrupt the oligomeric structure of the soluble Env glycoprotein preparations, as determined by cross-linking with 1 mM EGS and analysis by SDS-PAGE (4% gel) and Western blotting.

**Screening of hybridoma supernatants.** Hybridoma supernatants were screened by immunoprecipitation. Briefly, 100  $\mu$ l of culture supernatant was incubated with 100  $\mu$ l of PBS containing 0.5% Triton X-100, 0.5% Nonidet P-40, iodinated Env glycoprotein (approximately 100,000 cpm), and 8  $\mu$ g of rabbit anti-mouse immunoglobulin G (IgG; Calbiochem, La Jolla, Calif.) for 1 h at room temperature in microcentrifuge tubes. The oligomeric forms of Env used for screening each set were the same as that used for immunization. Protein A-Sepharose beads (100  $\mu$ l of a 20% [vol/vol] suspension) were then added, and tubes were rocked for 30 min. The beads were centrifuged, and the pellets were washed once with PBS containing 0.5% Triton X-100 and 0.5% Nonidet P-40. The tubes were counted in a Beckman Gamma 5500B counter. MAb 902 (10) and polyclonal rabbit antibody R160 (84) were included as positive controls; culture supernatant from an irrelevant hybridoma was included as a negative control.

Hybridoma colonies producing immunoglobulin which immunoprecipitated Env were expanded to approximately  $4 \times 10^6$  to  $5 \times 10^6$  cells and cloned by limiting dilution by standard methods. MAbs were produced as tissue culture supernatants in Iscove's minimal essential medium containing hypoxanthine, thymidine, 10% fetal calf serum, and 50 U of interleukin-6 per ml. In some instances, MAbs were produced in serum-free HyQ medium (HyClone) with 50 Units of interleukin-6 per ml. Mouse IgG subclass types were determined by using the AB-STAT typing kit (Sangstat Medical Co., Menlo Park, Calif.). Mouse IgG concentrations in tissue culture supernatants were determined with an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim Biochemicals).

**Metabolic labeling and immunoprecipitation.** BS-C-1 cells were infected with recombinant vaccinia virus at a multiplicity of infection of 20. At 4 h postinfection, the virus inoculum was replaced with methionine-free minimal essential medium con-

taining 5% dialyzed fetal calf serum and 100  $\mu$ Ci of [ $^{35}$ S]methionine per ml and incubated overnight. Cells were lysed in buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100. Soluble, secreted forms of Env glycoprotein were obtained from the medium of infected cells. For preparation of the ectodomain fragment of gp41 (gp41s), consisting of the N-terminal 172 amino acids of gp41, the medium of cells infected with vCB-14 was concentrated and applied to a 5 to 20% sucrose gradient as described above. The gp120 and gp41s dissociated from one another during centrifugation, and gp41s was recovered in a single peak sedimenting more slowly than monomeric gp120 (6). Monomeric gp120 was obtained from the medium of infected cells and in some cases was purified by sucrose density gradient centrifugation. Immunoprecipitations were performed by incubating metabolically labeled Env with antibody overnight at 4°C. Typically, 200  $\mu$ l of a hybridoma culture supernatant or 1  $\mu$ l of a polyclonal antiserum was used for each immunoprecipitation. Where appropriate, 8  $\mu$ g of rabbit anti-mouse IgG (Calbiochem) was then added for 30 min followed by 100  $\mu$ l of a 20% protein A-Sepharose suspension. After 30 min of rocking, the Sepharose beads were centrifuged at  $1,000 \times g$  for 4 min and the pellets were washed twice with 1 ml of Triton buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.1% Triton X-100). Proteins were eluted by heating for 5 min at 95°C in sample buffer containing 5% 2-mercaptoethanol.

**Western blotting.** Proteins from extracts of vaccinia virus-infected cells expressing different HIV-1 *env* genes were separated by SDS-PAGE (10% gel) and transferred to nitrocellulose membranes. In some cases, proteins were separated on preparative gels and the nitrocellulose was cut into 10-mm strips after protein transfer. The nitrocellulose membranes were incubated with MAb (usually 1:5 dilution) for 1 h at room temperature. After washing with PBS containing 0.2% Tween 20, the strips were incubated with  $^{125}$ I-labeled rabbit anti-mouse IgG for 30 min and then washed. Hybridization with polyclonal antibody R160 was done at 1:500 dilution, and  $^{125}$ I-protein A was used to detect binding. Proteins were visualized by autoradiography.

**Flow cytometry.** Human H9 cells ( $10^6/50 \mu$ l) chronically infected with HIV-1 IIIB were incubated with hybridoma supernatant. After 30 min at 4°C, the cells were washed twice with PBS containing 1% bovine serum albumin, incubated with goat anti-mouse-fluorescein isothiocyanate for 30 min at 4°C, and then washed two times. The cells were resuspended in 1 ml of PBS containing 4% paraformaldehyde and analyzed with a fluorescence-activated cell sorting (FACS) apparatus (FACScan; Becton Dickinson).

**Peptide ELISA.** The HIV-1 IIIB V3 loop peptide (CNTR KSIRIQRGPGRAFVTIGK) (American Bio-Technologies, Cambridge, Mass.) and the HIV-1 MN V3 loop peptide (YNKRKRIHIGPGRAFYTTKNIIG) (Biological Resources Branch, National Institute of Allergy and Infectious Diseases [NIAID]) were used to determine the V3 loop reactivities of the MAbs. Briefly, the wells of Immulon II 96-well assay plates were coated with 50  $\mu$ l of 0.05 M sodium carbonate (pH 9.5) containing 0.25  $\mu$ g of peptide overnight at 4°C. Plates were washed with PBS containing 0.1% Tween 20 and blocked with a solution of proteolyzed gelatin (Boehringer Mannheim Biochemicals). Antibody binding was performed at room temperature for 1 h. Serial dilutions were tested in duplicate. Bound MAb was detected with a peroxidase-conjugated anti-mouse IgG (Boehringer Mannheim Biochemicals) and 2,2'-amino-di-[3-ethylbenzthiazoline sulfonate(6)] substrate (Boehringer Mannheim Biochemicals). All MAbs exhibiting binding were

reexamined on mock-coated plates, and no false positives were detected.

**CD4 blocking.** To assay for CD4 blocking ability, metabolically labeled gp140 and sCD4 were prepared from the medium of cells infected with vPE12B and vCB-5, respectively. Dimeric gp140 was purified by sucrose density gradient centrifugation as described above. A 100- $\mu$ l sample of hybridoma supernatant (MAb in excess of Env) was incubated overnight at 4°C with dimeric gp140. A small amount of sCD4 was added, and the mixture was incubated for 30 min at room temperature. Then 4  $\mu$ g of rabbit anti-mouse IgG was added for 30 min, followed by 100  $\mu$ l of protein A-Sepharose beads (20% suspension). After 30 min of gentle rocking, the beads were washed once with buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100, and samples were analyzed by SDS-PAGE (10% gel). MAb F105 (67), obtained from the AIDS Research and Reference Program, NIAID, was used as a positive control for CD4 blocking activity. Anti-V3 loop MAb 902 (10) was used as a negative control.

## RESULTS

**Purification of secreted oligomeric Env protein.** Secreted oligomeric HIV-1 Env protein was produced by recombinant vaccinia viruses, vCB-14 and vPE12B, containing mutated *env* genes. In both constructs, the genes were truncated after amino acid 678 (Lys), just prior to the transmembrane domain, resulting in 140-kDa Env molecules (gp140). To prevent dissociation of gp120 from the gp41 ectodomain fragment during purification, a deletion was made in the DNA sequence coding for the amino acids at the gp120-gp41 cleavage site in vPE12B. We have previously shown that this mutation, introduced into the full-length *env* gene, yielded a noncleaved form of Env that was efficiently folded, assembled, and transported to the plasma membrane where it could bind to added sCD4 (17). In both constructs, a synthetic early/late vaccinia virus promoter was used to allow high levels of gene expression (7). Both proteins were efficiently secreted and could bind CD4 (not shown), suggesting that they were folded correctly. As designed, the protein made by vCB-14-infected cells was secreted in both cleaved and noncleaved forms, while that expressed by vPE12B was recovered primarily as noncleaved gp140. Although the primary and secondary proteolytic cleavage sites of Env were removed in vPE12B, a very small amount of cleavage occurred, as was observed with the full-length form (17).

To produce oligomeric gp140 for immunization, BS-C-1 monolayers were infected with vPE12B and incubated 24 to 36 h in serum-free medium. The gp140 was purified from the medium by a two-step procedure. First, glycoproteins from the medium were bound to a lentil lectin column and eluted with methyl  $\alpha$ -D-mannopyranoside. This step resulted in elimination of most contaminating proteins (Fig. 1C, lane 2). After concentration, oligomeric and monomeric gp140 were separated by sucrose velocity gradient sedimentation. The gradients were fractionated, and a small aliquot of each fraction was analyzed by SDS-PAGE and Western blotting to monitor the distribution of gp140 (Fig. 1A). The majority of the Env glycoprotein was in dimeric and tetrameric forms. A minor peak containing monomeric gp140 and gp120 was also obtained.

Peak fractions containing the three forms were pooled separately, and an aliquot of each was chemically cross-linked with EGS to confirm their oligomeric states. As shown in Fig. 1B, dimeric and tetrameric Env fractions were cross-linked into dimers and larger forms, respectively, whereas monomeric

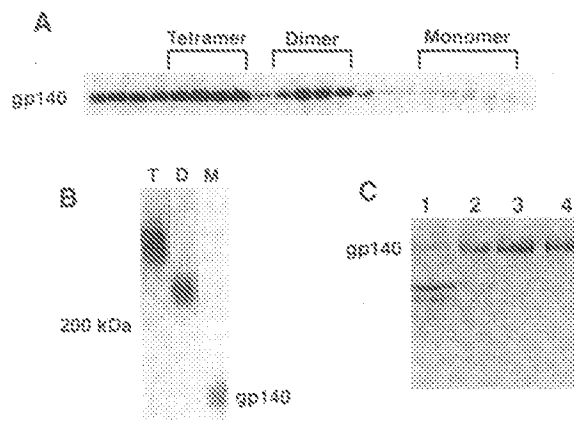


FIG. 1. Purification of monomeric and oligomeric forms of soluble, noncleaved gp140. (A) After separation by lentil lectin chromatography, glycoproteins from the medium of vPE12B-infected cells were separated by sucrose velocity gradient centrifugation. A portion of each gradient fraction was separated by SDS-PAGE (10% gel) and analyzed by Western blotting. Tetramer, dimer, and monomer fractions, as indicated, were pooled. (B) An aliquot of each pool was cross-linked with EGS, separated by SDS-PAGE (4% gel), and analyzed by Western blotting. (C) Proteins from each purification step were analyzed by Coomassie blue staining. Lanes: 1, medium of infected cells; 2, lentil lectin eluate; 3 and 4, dimer and tetramer fractions, respectively, from sucrose density gradient.

Env was not cross-linked into larger-molecular-weight species. Similar results have been demonstrated with full-length Env (14). Analysis of the Coomassie blue staining pattern of each sucrose gradient peak indicated that gp140 was the predominant band in all three preparations, although monomeric Env was less pure than either oligomeric form (Fig. 1C and data not shown).

**Immunization of mice and production of MAbs.** Two mice were immunized with monomeric, three were immunized with dimeric, and four were immunized with tetrameric gp140. We found that emulsification of the preparations in RiBi adjuvant did not affect the oligomeric state of the Env, as shown by chemical cross-linking followed by Western blot analysis (not shown). Serum collected from each animal after the first inoculation efficiently reacted with gp140 by immunoprecipitation and with gp160, gp120, and gp41 by Western blotting (not shown). The mice were sacrificed 3 days after the final inoculation, and hybridomas were generated. To identify antibodies capable of recognizing conformation-dependent epitopes, hybridoma supernatants were tested for the ability to immunoprecipitate iodinated, gradient-purified oligomeric or monomeric gp140. The form of Env used for screening was the same as the form used for immunization. The results of this screening procedure were unambiguous. Radioactivity determinations were <1,000 cpm for negative wells and >20,000 cpm for positive wells. During the initial screening, random samples that were deemed positive were analyzed by SDS-PAGE to ensure that gp140 was indeed immunoprecipitated. No false positives were detected. A total of 190 hybridomas from nine mice in five fusion experiments were strongly positive in this assay. Of these, 180 were still positive after expansion, and so far 138 have been cloned by limiting dilution. Of the 138 cloned hybridomas, only 15 were derived from mice immunized with monomeric Env. Several factors could account for this relatively low yield. Although approximately the same amount of Env protein was used to immunize each mouse, the

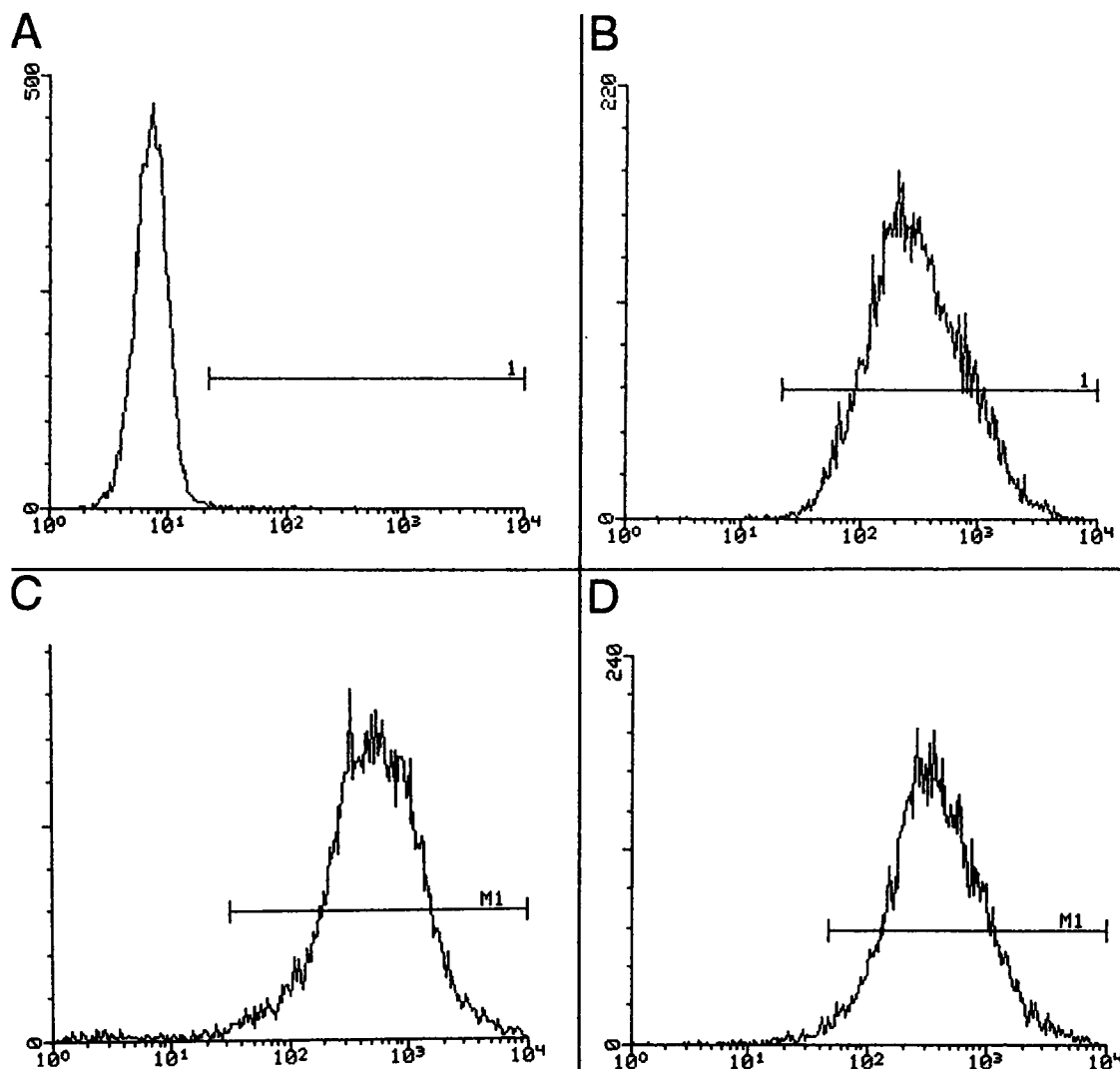


FIG. 2. FACS profiles of MAb. Human T cells chronically infected with HIV-1 IIIB were incubated with hybridoma supernatant followed by goat anti-mouse-fluorescein isothiocyanate and subjected to FACS analysis. (A) Nonspecific mouse IgG; (B) anti-gp120 MAb 110.4; (C) anti-gp120 MAb D34; (D) anti-gp41 MAb D6.

monomer preparation was less pure than the dimer and tetramer preparations, resulting in immunization with some extraneous proteins. A more interesting possibility, however, is that monomeric Env is less immunogenic than oligomeric Env. Studies to address this point directly are under way. Hybridomas, and the MABs that they secrete, were first designated M (monomer), D (dimer), or T (tetramer), depending on the immunogen used, and numbered sequentially after isolation. The IgG subtypes were determined for 128 of the MABs; 62 were IgG1, 52 were IgG2a, 13 were IgG2b, and 1 was IgG3.

**Reactivity of MABs to native HIV-1 Env.** Because the immunogen used was a noncleavable, truncated form of Env, it was important to ascertain whether the MABs recognized the authentic HIV-1 Env molecule. To do this, approximately 90% of the MABs were screened by FACS analysis for the ability to recognize Env on the surface of cells chronically infected with HIV-1 IIIB. Representative FACS profiles are shown in Fig. 2. A control mouse IgG did not label the cells (Fig. 2A), while a previously described MAB to gp120, 110.4 (Fig. 2B), labeled the cells strongly. The profiles of one new anti-gp120 MAB,

D34, and one new anti-gp41 MAB, D6, are shown in Fig. 2C and D, respectively. At least 80% of the MABs were clearly positive in this assay. Lack of reactivity could be due to a very low titer of antibody or reactivity with an epitope that is unique to the recombinant gp140. However, the fact that the large majority of the MABs tested recognized native HIV-1 Env provides strong evidence that the recombinant, oligomeric gp140 used here faithfully reflects the antigenic structure of the authentic molecule.

**Identification of conformation-dependent and -independent MABs.** To determine which MABs recognized linear, conformation-independent epitopes, each was screened for the ability to react by Western blot analysis with Env that had been denatured and reduced prior to SDS-PAGE. A representative panel is shown in Fig. 3. We found that 43% of the MABs reacted strongly with Env, 11% reacted very weakly (e.g., D9 and D59), and 46% were completely negative even though they efficiently immunoprecipitated Env. We defined MABs that reacted either very weakly or not at all by Western blotting as conformation dependent. The remaining MABs, which reacted

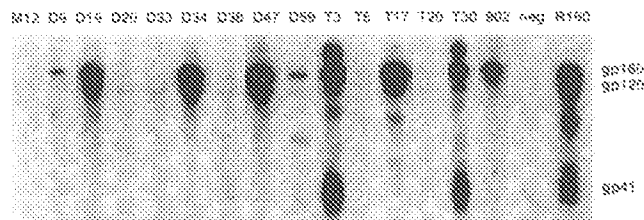


FIG. 3. Western blot analysis of MAb reactivity. A cell lysate of BS-C-1 cells infected with vPE16 was separated by preparative SDS-PAGE (10% gel) and transferred to nitrocellulose. Strips of nitrocellulose membrane were incubated with individual MAbs. All 138 MAbs were tested in this way; a representative set is shown. MAbs were classified as conformation dependent if they showed no reactivity (for example, M12 and D20) or weak reactivity (D9 and D59) and as conformation independent if they were strongly reactive (for example, D19 and D34). The subunit recognized by the conformation-independent MAbs was also determined by this analysis. For example, T3 and T30 recognize gp41, while D19, D34, D47, and T17 recognize gp120.

strongly with denatured Env, were defined as conformation independent. By this criterion, 57% (79 of 138) of the MAbs recognized conformationally sensitive antigenic epitopes, while 43% (59 of 138) recognized linear or conformation-independent epitopes (Table 1). Thus, the immunization and screening approach used here generated a large number of antibodies, more than half of which recognized conformation-dependent epitopes.

**Subunit mapping.** The epitope recognized by each MAb was mapped initially to either gp120 or the gp41 ectodomain. Mapping of the conformation-independent MAbs was done by

Western blot analysis as shown in Fig. 3. Of the 59 conformation-independent MAbs analyzed, 50 recognized epitopes in gp120 and 9 recognized epitopes in gp41 (Table 1). Subunit mapping of the conformation-dependent MAbs was performed by immunoprecipitation analyses using several different metabolically labeled forms of Env (Fig. 4). These included a cell lysate containing full-length gp160, gp120, and gp41 (Fig. 4A), monomeric gp120 (Fig. 4B), and a gradient-purified gp41 ectodomain fragment derived from vCB-14 (Fig. 4C). The results obtained from a panel of conformation-dependent MAbs is shown in Fig. 4A to C. Of the 79 conformation-dependent MAbs analyzed, 33 mapped to gp120 (Table 1). Many of these anti-gp120 MAbs coprecipitated gp41 in a lysate containing gp160, gp120, and gp41 (for example, D20; Fig. 4), presumably because of noncovalent interactions. However, none of the anti-gp41 MAbs coprecipitated gp120 either because the gp120-gp41 complex was not reactive or because it was dissociated by the MAb.

Of the MAbs that did not immunoprecipitate purified gp120, many immunoprecipitated the purified gp41 ectodomain fragment, indicating that their epitopes reside in the gp41 ectodomain (for example, D12 and D40; Fig. 4C). However, a number of MAbs were unable to immunoprecipitate either purified gp120 or the gp41 ectodomain fragment by itself. These MAbs, such as T6, immunoprecipitated both gp41 and gp160 when they were together in cell lysates (Fig. 4A). As a consequence, it was not possible to determine whether these MAbs recognized epitopes unique to gp160 and coimmunoprecipitated gp41 or whether they recognized oligomer-dependent epitopes present in gp41. To investigate these possibilities further, the MAbs were tested for the ability to immunoprecipitate a chimeric Env protein consisting of HIV-2 gp120 and HIV-1

TABLE 1. Reactivities of 138 MAbs raised against soluble forms of the HIV-1 Env protein<sup>a</sup>

MAb	Total	Conformation-independent epitope	Conformation-dependent epitope	V3 loop	CD4 blocking
All					
Total	138	59	79	15/82	19/76
Conformation dependent	79			0/32	19/49
Conformation independent	59			15/50	0/27
gp120					
Total	83	50	33	15/82	19/41
Conformation dependent	33			0/32	19/20
Conformation independent	50			15/50	0/21
gp41					
Total	52	9	43		0/35
Conformation dependent	43				0/29
Conformation independent	9				0/6
Immunogen					
Monomer					
All	15	9	6		3/12
gp120	12	9	3	7/12	3/10
gp41	3	0	3		0/2
Dimer					
All	68	19	49		14/41
gp120	34	14	20	6/33	14/22
gp41	31	5	26		0/19
Tetramer					
All	55	31	24		2/23
gp120	37	27	10	2/37	2/9
gp41	18	4	14		0/14

<sup>a</sup> MAbs are classified on the basis of which subunit they recognize (gp120 or gp41), whether they recognize conformation-dependent or -independent epitopes, and by the immunogen used to elicit them. The subunit recognized by three conformation-dependent MAbs made with dimeric gp140 as immunogen has not been determined. Reactivity against the V3 loop and ability to block CD4 binding were determined by peptide ELISA or immunoprecipitation analysis, respectively. Not all MAbs have been tested for reactivity with the V3 loop and CD4 blocking; thus, the total number of positive MAbs over the total number tested is shown.

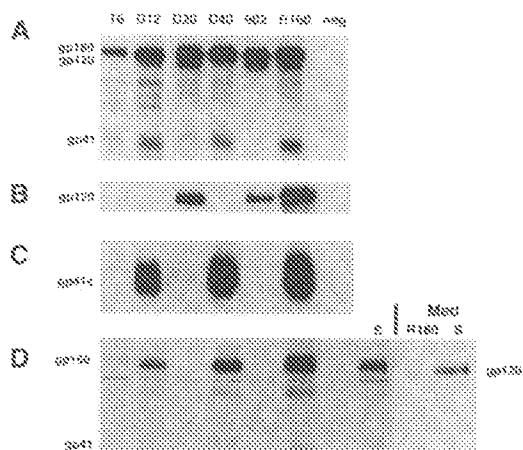


FIG. 4. Subunit mapping of conformation-dependent MABs. The subunit recognized by each conformation-dependent MAb was identified by immunoprecipitation analysis with different forms of Env. For all panels, metabolically labeled Env proteins were produced by infection of BS-C-1 cells with a recombinant vaccinia virus expressing a form of Env. The rabbit polyclonal anti-gp160 antibody R160 (84) was used to show all forms of Env in a given sample. Anti-V3 loop MAb 902 (9) was used as an anti-gp120 positive control, and an irrelevant MAb was used as a negative (neg) control. Each panel shows immunoprecipitation with a different form or preparation of Env as follows: (A) a lysate of cells infected with vSC60 containing wild-type gp160 and the proteolytic cleavage products gp120 and gp41; (B) the medium of cells infected with vPE16 containing monomeric gp120; (C) the ectodomain fragment, gp41s, prepared by sucrose density gradient purification of the medium of cells infected with vCB-14 which expresses cleavable gp140; and (D) a chimera consisting of HIV-2 gp120 and HIV-1 gp41 expressed by vSC64 (7). An extract of cells infected with vSC64 was subjected to immunoprecipitation by the set of MABs as well as by anti-simian immunodeficiency virus monkey serum (S) (donated by V. Hirsch, NIAID). The sharp band migrating slightly below the position of gp120 may be due to  $\beta$ -galactosidase, which is coexpressed by the recombinant vaccinia viruses. The gp160 band appears as a doublet in cells infected with vSC64 (D) as well as in cells infected with vSC50 which expresses HIV-2 Env (not shown). In addition, gp120 (HIV-2) from the medium (Med) of cells infected with vSC64 was immunoprecipitated with R160 and anti-SIV serum (D).

gp41 (vSC64). None of the MABs in the class represented by T6 immunoprecipitated HIV-2 Env (not shown). They did, however, recognize the chimeric Env protein (Fig. 4D), indicating that the epitopes to which they bind are present in the HIV-1 gp41 ectodomain.

Taken together, more than one-third of the MABs derived from immunization with native oligomeric Env protein were directed against epitopes in the gp41 ectodomain. Comparison of the subunit mapping results with the data on conformation dependence revealed that the antigenic structure of gp41 is exquisitely sensitive to conformation. More than 80% (43 of 52) of the MABs to gp41 recognized conformation-dependent epitopes (Table 1). By contrast, the antigenic sites of gp120 appeared to be less sensitive to tertiary structure, since 40% (33 of 83) of the gp120 MABs recognized conformational epitopes (Table 1).

**Detailed epitope mapping of MABs.** To map the epitopes recognized by the MABs more precisely, a series of C-terminally truncated Env molecules was used. These were expressed either by recombinant vaccinia viruses or by the transient vaccinia virus-T7 system (21). The Env molecules used included full-length gp140 (678 amino acids), two molecules with

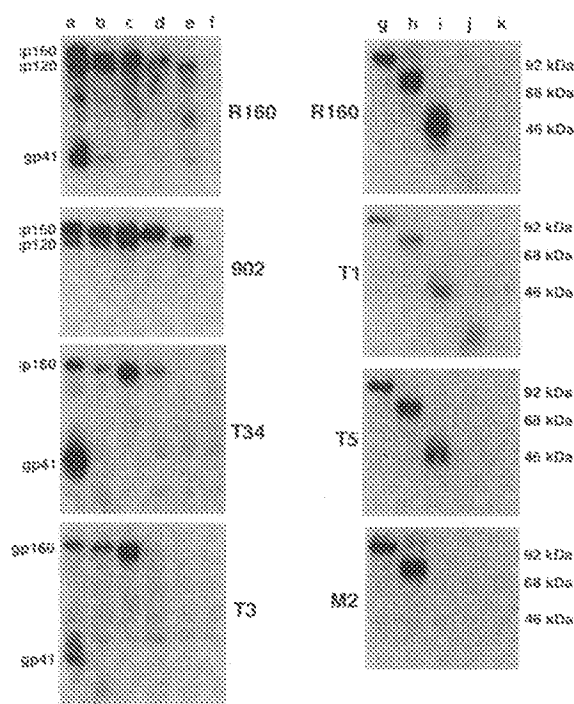


FIG. 5. Epitope mapping of the conformation-independent MABs by Western blot analysis. A set of C-terminally truncated Env molecules expressed by vaccinia viruses was used to determine the region of Env with which each conformation-independent MAB reacted. Extracts of cells expressing the truncated Env proteins were separated by SDS-PAGE (10% gel) and blotted to nitrocellulose. The MABs with reactivity to gp41 were mapped with gp160 (lane a) and four sequential truncations in gp41 containing 747 (lane b), 678 (lane c), 635 (lane d), and 574 (lane e) amino acid residues. The MABs with reactivity to gp120 were mapped with gp120 (lane g) and three sequential truncations in gp120 containing 393 (lane h), 287 (lane i), and 204 (lane j) amino acid residues. The virus vSC8, which does not express Env protein, was used as a negative control (lanes f and k). Antibody names are given between the vertical panels of blots.

sequential truncations in gp41 (635 and 574 amino acids), full-length gp120 (502 amino acids), and three truncated forms of gp120 (393, 287, and 204 amino acids) (17, 19). Mapping of the conformation-independent MABs was performed by Western blotting using extracts of cells expressing the truncated Env molecules. The map location is indicated by the first segment removed that results in loss of reactivity. Thus, T34, T3, T1, T5, and M2 map to locations progressively closer to the N terminus of gp160. Representative examples of Western blots are shown in Fig. 5, and the results for all MABs tested are summarized in Fig. 6. Of the nine anti-gp41 MABs tested, one mapped to amino acids 503 to 574, five mapped to amino acids 575 to 635, and three mapped to amino acids 636 to 678. Of the 50 anti-gp120 MABs to conformation-independent epitopes, 32 mapped to the amino-terminal 204 amino acids, 3 mapped to amino acids 204 to 287, and 15 mapped to amino acids 287 to 393. Somewhat surprisingly, no conformation-independent MABs mapped to the C-terminal region of gp120 (between amino acids 393 and 502) even though antibodies to this region are abundant in human sera (2, 37, 41, 45, 64). This could be due to sequestration of the C terminus of gp120 by interactions with adjoining Env subunits or with gp41. Alternatively, the conformation of the uncleaved gp140 used as immunogen could mask this region.

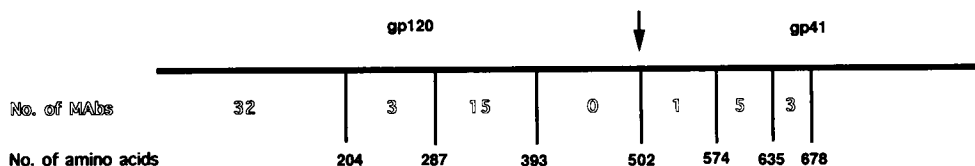


FIG. 6. Compilation of the reactivities of the 59 conformation-independent MABs with truncated Env molecules. Each of the MABs was analyzed for reactivity by Western blot analysis using a set of C-terminally truncated Env molecules as shown in Fig. 5. The number of amino acids in each truncated Env molecule and the number of MABs mapping to each region are given.

Initial mapping of the conformation-dependent MABs was done by immunoprecipitation of metabolically labeled, truncated Env molecules. Of the 39 anti-gp41 MABs tested, 3 efficiently immunoprecipitated the 635-amino-acid Env molecule, indicating that amino acids 635 to 678 are not necessary for antibody recognition. Of the 32 anti-gp120 antibodies tested, only 2 immunoprecipitated the 393-amino-acid gp120 molecule; one of these also immunoprecipitated the 287-amino-acid molecule. However, the inability of a conformation-dependent MAB to immunoprecipitate a truncated form of Env does not necessarily imply that its epitope lies completely or even partially within the truncated area, since the overall structure of the truncated molecules may be significantly different from the native, full-length protein. Other approaches are being used to map the epitopes recognized by our panel of conformation-dependent antibodies.

**Reactivity of MABs with the V3 loop peptide.** To determine the fraction of MABs directed against the V3 loop generated by immunization with oligomeric Env glycoprotein, a V3 loop peptide ELISA assay was performed with anti-gp120 MABs. We found that 15 of 50 conformation-independent MABs exhibited reactivity with the HIV-1 IIIB V3 loop peptide (Table 1). Of these, two cross-reacted with a V3 loop peptide from the MN strain. Similar cross-reactive antibodies to the V3 loop have been reported previously (25, 27, 60). In addition, an interesting correlation was observed between the oligomeric state of the immunogen used and the frequency with which anti-V3 loop MABs were derived. Of the 15 MABs derived from animals immunized with monomeric gp140, 7 were against the V3 loop. In contrast, only 6 of 68 MABs from animals immunized with dimer and 2 of 55 immunized with tetramer bound to the V3 loop peptide (Table 1). Thus, a far greater proportion of non-V3 loop MABs was obtained when oligomeric Env was used as the immunogen, indicating that the V3 loop may not be a predominant epitope when presented in the context of oligomeric Env. Immunization with oligomeric Env may, as a consequence, generate a greater proportion of antibodies to conserved, conformational epitopes rather than to variable, linear regions of the protein.

**CD4 blocking ability of MABs.** Unlike antibodies to the V3 loop, neutralizing antibodies which block Env-CD4 binding generally recognize conformationally sensitive epitopes and often recognize the Env from divergent strains (8, 11, 34, 36, 41, 46, 67, 77). We tested a large panel of MABs to both gp120 and gp41 for the ability to block binding of sCD4 to Env. MABs were incubated overnight with gradient-purified, metabolically labeled dimeric gp140 derived from vPE12B. The amount of antibody used was determined to be in excess over the amount of Env. Metabolically labeled sCD4 was then added for 30 min followed by rabbit anti-mouse IgG and protein A-Sepharose beads. Coimmunoprecipitation of sCD4 by the anti-Env MABs was monitored by SDS-PAGE. MAB F105 (67), which blocks CD4 binding, and the anti-V3 loop MAB 902 (10), which does not, were used as controls. A representative set of results is

shown in Fig. 7. MAB D20, for example, efficiently blocked sCD4 binding, while D16 did not. As expected, none of the anti-gp41 MABs blocked binding of sCD4 regardless of their conformation dependence. However, of the 20 conformation-dependent anti-gp120 MABs tested, 19 efficiently blocked sCD4 binding (Table 1). In contrast to conformation-dependent anti-gp120 MABs, none of the 21 conformation-independent anti-gp120 MABs tested blocked CD4 binding. Thus, in this panel of MABs, the ability to block sCD4 binding was restricted to conformation-dependent antibodies to gp120.

## DISCUSSION

Characterizing the structural determinants that affect the HIV-1 Env glycoprotein antigenic structure is important for designing Env subunit preparations capable of eliciting broadly cross-reactive, neutralizing antibodies as well as for understanding the humoral response to HIV-1 infection. It is becoming increasingly apparent, for example, that antibodies to conformational epitopes comprise an important component of the immune response to the Env glycoprotein (30, 43, 53, 76). One conformational determinant which may influence Env immunogenicity is its quaternary structure. Like most other viral membrane proteins, Env assembles into oligomers in the endoplasmic reticulum (20), and by analogy with other viral and cellular membrane proteins, assembly is apt to be a prerequisite for further transport (13, 72, 84). To determine the effects of quaternary interactions on Env antigenic structure, we isolated oligomeric Env for immunization and used a screening technique which would allow us to identify antibodies that reacted with non-denatured molecules. To enable purification that would not disrupt the oligomeric structure of the protein, we designed a secreted form of Env which contained all of gp120 and the entire gp41 ectodomain. To prevent dissociation of gp120 from the gp41 ectodomain during isolation, a mutation was introduced to preclude cleavage of the precursor molecule. We and others have shown that cleavage is not essential for conformational maturation and transport (3, 17, 31). The resulting molecule, noncleaved

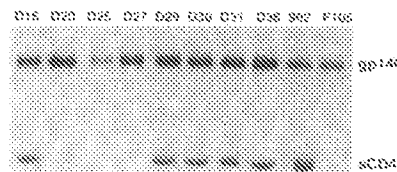


FIG. 7. Analysis of the CD4 blocking ability of MABs. Metabolically labeled, gradient-purified dimeric gp140 was incubated with an excess of MAB overnight. Then trace amounts of metabolically labeled sCD4 were added for 1 h, and immunoprecipitation was performed. MABs F105 and 902 were used to show CD4 blocking and lack of blocking, respectively.

gp140, was secreted primarily in oligomeric form, consistent with reports that have shown the ectodomain of gp41 to be largely responsible for subunit assembly, though interactions between adjoining gp120 subunits may occur (19, 62, 81).

Several lines of evidence suggest that secreted, oligomeric gp140 accurately reflected native Env structure. First, the protein was efficiently secreted. Since misfolded molecules are typically retained in the endoplasmic reticulum and degraded (13, 39, 72), it is unlikely that gp140 was grossly misfolded. Second, gp140 bound sCD4 as well as the conformation-dependent MAb F105 efficiently. Because the CD4 and F105 binding sites in gp120 are discontinuous in nature (44, 61, 67), the gp120 portion of the molecule appeared to be relatively intact. However, the strongest evidence that purified gp140 accurately reflects native Env structure came from analysis of the antibodies raised against it in this study. The large majority of MAbs recognized Env on the surface of chronically infected cells, and 79 of the 138 MAbs recognized conformational epitopes in either gp120 or gp41. These results demonstrate that when oligomeric Env is used for immunization, antibodies to conformation-dependent epitopes are readily generated and suggest that Env tertiary and quaternary structure are important determinants for the humoral response. The large number of MAbs to conformational epitopes reported here lend further support to the idea that oligomeric protein elicits a qualitatively different immune response than monomeric protein (whether native or denatured) and show that it is possible to generate a soluble, oligomeric form of Env which reflects the native structure of the wild-type protein. Several other groups have reported small panels of MAbs produced by immunization with a soluble, secreted form of Env similar to the one that we have used (56, 65). However, relatively few conformation-dependent MAbs were obtained, most likely because of the use of ELISA screening procedures that may preferentially recognize unfolded protein.

Preliminary epitope mapping of the 83 MAbs directed against gp120 revealed several interesting features. We noted that antibodies to the V3 loop comprised a high percentage of MAbs generated from animals immunized and screened with monomeric Env compared to oligomeric Env. Thus, 47% (7 of 15) of the MAbs from animals immunized with monomeric Env bound to epitopes in the V3 loop, compared with 7% (8 of 123) of the MAbs derived from mice immunized with oligomeric protein. This finding suggests that the V3 loop is not as immunogenic in the context of oligomeric protein as it is in monomeric protein. This is consistent with recent studies which have shown that antibodies to the V3 loop are not prevalent in human sera (32, 53). While anti-V3 loop antibodies can be easily generated in the laboratory by immunization with peptides or protein and detected in human sera by using similar material in ELISA assays (25–28, 40, 47, 52, 56, 58–60, 63, 69, 74, 76), the contribution of the V3 loop in the overall immune response to native oligomeric Env may not be great.

The C-terminal 109 residues of gp120 comprised another region to which relatively few antibodies were directed. None of the 50 antibodies to linear gp120 epitopes mapped here, which was surprising since this region contains an immunodominant epitope to which antibodies can be detected in human sera (37, 41, 45, 64). One explanation for our results is that this region may be partially sequestered in the oligomeric molecule through interactions with adjoining gp120 subunits or gp41. In fact, there are reports which suggest that the C-terminal region of gp120 interacts with gp41 (33, 50). Antibodies in human sera to the C-terminal region of gp120 might arise from the presence of shed, monomeric gp120 which was absent from our oligomeric Env preparations. Though present in

human sera, these antibodies may not react or react weakly with oligomeric Env on the surface of virions or infected cells. The fact that these antibodies do not neutralize virus or have weak neutralizing activity (41, 45, 53, 64) is consistent with this model. An alternative explanation for the absence of MAbs to the C terminus of gp120 is that this region is buried in the uncleaved gp140 molecule used for immunization. This possibility cannot be ruled out by the data presented here. Finally, a large number of conformation-dependent anti-gp120 MAbs were found to block sCD4 binding, consistent with reports that indicate gp120 and CD4 interact over a large area representing several discontinuous regions of gp120 (8, 11, 35, 36, 41, 46, 53, 56, 61, 67, 76, 77, 79). By contrast, none of the conformation-independent anti-gp120 MAbs or anti-gp41 MAbs blocked CD4 binding.

Because of the efficiency with which gp140 assembled, it was not possible to obtain large amounts of monomeric gp140. As a consequence, we cannot determine whether the small number of MAbs generated from animals immunized with monomeric gp140 was due to a relatively weak immune response to the monomeric molecule or to differences resulting from less pure protein. While additional studies will be required to determine whether oligomeric Env elicits a stronger humoral response than that seen with monomeric Env, our results do demonstrate several important qualitative differences. A very strong immune response to gp41 was obtained by immunization with oligomeric Env, as judged by the percentage of anti-gp41 MAbs identified. The gp41 ectodomain contained 26% of the total amino acid content of the protein, and 38% (52 of 138) of the MAbs recognized epitopes in this region. Analysis of the anti-gp41 MAbs showed that 82% (43 of 52) recognized conformationally sensitive epitopes, a much higher proportion than that seen with the anti-gp120 MAbs. Furthermore, we have recently found that 60% (21 of 35) of the MAbs which recognize gp41 react more strongly with oligomeric than monomeric Env or, in some instances, are oligomer specific. In addition, some of these MAbs possess neutralizing activity (6). These results suggest that the antigenic structure of gp41 is critically dependent on both Env tertiary and quaternary structure and that the two cannot be dissociated from one another. In addition, we have found that a large number of our MAbs directed against gp120 react more strongly with monomeric than oligomeric protein (6). Thus, immunization with gp120, even if it retains native structure, may lead to the generation of antibodies which react poorly with oligomeric Env on virions and the surface of infected cells.

In summary, we have constructed a soluble, oligomeric form of the HIV-1 Env glycoprotein which reflects native Env structure and elicits a diverse array of antibody reactivities, particularly antibodies to conformational epitopes. The repertoire of antibodies raised against oligomeric Env is qualitatively different than that raised against monomeric Env. It is clear, for example, that Env quaternary structure has significant antigenic implications both in gp41 and gp120. The large number of MAbs that we have generated against gp41, all of which immunoprecipitate native protein, should make it possible to construct a relatively detailed antigenic map of this subunit and to identify regions that are immunogenic and conserved and to which neutralizing antibodies are directed. These findings, coupled with observations that native Env protein elicits neutralizing antibodies more effectively than the denatured molecule (30, 75), strongly argue that taking into account Env quaternary structure will be important in understanding the humoral response to HIV-1 infection and potentially for the design of Env subunit preparations which can effectively elicit broadly cross-reactive, neutralizing antibodies.

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## EXHIBIT 3

# Mapping the Protein Surface of Human Immunodeficiency Virus Type 1 gp120 using Human Monoclonal Antibodies from Phage Display Libraries

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Panels of hybridoma-derived monoclonal antibodies against diverse epitopes are widely used in defining protein surface topography, particularly in the absence of crystal or NMR structural information. Here we show that recombinant monoclonal antibodies from phage display libraries provide a rapid alternative for surface epitope mapping. Diverse epitopes are accessed by presenting antigen to the library in different forms, such as sequential masking of epitopes with existing antibodies or ligands prior to selection and selection on peptides. The approach is illustrated for a recombinant form of the human immunodeficiency virus type 1 (HIV-1) surface glycoprotein gp120 which has been extensively mapped by rodent and human monoclonal antibodies derived by cellular methods. Human recombinant Fab fragments to most of the principal epitopes on gp120 are selected including Fabs to the C1 region, a C1/C5 epitope, a C1/C2 epitope, the V2 loop, the V3 loop and the CD4 binding domain. In addition an epitope linked to residues in the V2 loop and CD4 binding domain is identified.



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**Keywords:** human antibody repertoires; epitope mapping; HIV infection; combinatorial libraries; gp120 topology

## Introduction

Some proteins do not yield readily to structural solution by the classical approaches of crystallography or nuclear magnetic resonance (NMR) spectroscopy. The surface glycoprotein gp120 of the human immunodeficiency virus type 1 (HIV-1) is such a protein. Crystallization is hindered by its high carbohydrate content (about 50%) and NMR

structural studies by its relatively large size. Still there is an urgent need for structural information on the molecule. For instance such information would be valuable in understanding the nature of the gp120-CD4 and gp120-chemokine receptor interaction which is key to viral entry in to cells. Furthermore the molecule is important in eliciting neutralizing antibodies and so its structure has many implications for vaccine design.

Comparison of gp120 sequences from different HIV-1 strains has identified five variable domains (V1 to V5; Modrow *et al.*, 1987; Starcich *et al.*, 1986), of which the first four form disulphide-stabilized loops, and five conserved domains (C1 to C5). Computer modeling has further been used to suggest the location of secondary structural elements in gp120 (Gallaher *et al.*, 1995). Detailed

H. J. Ditzel and P. W. H. I. Parren contributed equally to this work.

Abbreviations used: HIV-1, human immunodeficiency virus type 1; gp, glycoprotein; Ig, immunoglobulin AP; AP, alkaline phosphatase; CD4bd, CD4 binding domain; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

information on the tertiary and quaternary structures of the native protein, however, are unavailable.

The surface topography of a protein can be examined through the study of panels of monoclonal antibodies reactive with the protein. Whilst such a study provides a view at far lower resolution than crystallography or NMR it can nevertheless be useful. For example, using a large panel of mostly rodent and some human monoclonal antibodies (mAbs), a low resolution model of gp120 has been constructed (Moore *et al.*, 1993b; Moore *et al.*, 1994a,b; Wyatt *et al.*, 1992; Moore & Sodroski, 1996).

Phage display libraries provide a rapid route to large numbers of mAbs from immune donors (Burton & Barbas, 1994; Burton *et al.*, 1991). In the case of recombinant monomeric gp120 as the selecting antigen, antibodies of notable sequence diversity have been retrieved but the great majority are directed to a series of related epitopes on the CD4 binding domain (CD4bd) of gp120 (Barbas *et al.*, 1993). In part this probably reflects the fact that the CD4bd is a major target for serum antibodies to gp120 in HIV-1 seropositive individuals (Moore & Ho, 1995). The relative conservation of this site is likely to be another factor favoring the observed bias to this site since the great variability of HIV-1 means that the libraries are challenged with a gp120 different to the immunizing antigen (we used mostly gp120 from the LAI strain for library selection). Epitopes associated with the variable loops, for example, are less conserved than CD4bd-associated epitopes so it is less likely that Fabs to the variable regions of gp120 will be isolated when heterologous gp120 is used for selection.

## Results

Previously, eight HIV-1 libraries have been panned on recombinant gp120 coated directly to microtiter wells which resulted in the isolation of a panel of Fab fragments specific for the gp120 CD4bd. Additional Fab fragments directed against a CD4bd/V2 loop-sensitive epitope have been retrieved after masking of CD4bd epitopes with an anti-CD4bd mAb.

### Epitope masking by capturing the antigen using antibody or ligand

The first strategy employed masking of CD4bd epitopes by capturing gp120 either by soluble CD4 or an anti-gp120 CD4bd mAb immobilized on solid phase. Selection of the libraries on soluble CD4-captured gp120 resulted in the isolation of three novel Fab fragments (Fab p7, p20 and p35). Panning on gp120 captured by the anti-CD4bd mAb yielded ten additional Fab fragments (Fab L15, L17, L19, L25, L34, L35, L52, L59, L69 and L81). The specificity of the different Fab fragments was demonstrated by their strong ELISA reactivity with gp120, but not with ovalbumin, human Fc fragment, transferrin or bovine serum albumin (BSA). The 13 Fab fragments were demonstrated to be diverse by sequence analysis of the variable regions of the heavy and light chains. As shown in Figure 1, the sequences of the heavy chain CDR3s were unrelated except for Fab L59 and L69, for which the whole heavy chain variable domain sequences differed by only seven amino acid residues from one another and which therefore may be somatic variants.

To determine which epitopes are recognized by the Fabs, we assessed their binding to a panel of HXBc2 gp120 mutants expressed in COS-1 cells. Binding of Fab p7, p20 and p35 revealed that all three Fabs are directed to closely related epitopes located in the N-terminal region of gp120. As shown in Figure 2(a), the binding of Fab p7 was completely abolished by amino acid substitution 45 W/S in the C1 region. Binding of Fab p7 was further markedly reduced by amino acid substitution 40 Y/D, and showed some dependency on substitutions at the C terminus of gp120, as demonstrated by decreased or enhanced binding by substitutions 475 M/S and 493 P/K. Very similar mutant maps were found for Fabs p20 and p35 (not shown).

The Fabs selected on gp120 captured by the anti-CD4bd mAb recognize four distinct epitope clusters. The majority of Fabs (i.e. L19, L34, L35, L52, L59, and L69) recognize a C1 epitope very similar to that recognized by Fabs p7, p20 and p35, as described above. A second epitope involving the C1 and C5 regions is recognized by Fab L81 (Figure 2). The binding of Fab L81 is abolished by a substitution in the C1 region (45 W/S)

Fab	FR3	CDR3	FR4
L15	TAVYYCAR	DSPGYSNTWYDWFEF	WGQGTLLTVVSS
L17	TAVYYCAT	GRPRWWQRDAFHF	WGQGTKVTVSS
L19	TAVYYCAR	HSGRYINGNYHPYGMDV	WGQGTTLTVVSS
L25	DARYYCAR	AWEVRIDHRYFFDL	WGQGTLLTVVSS
L34	TAVYYCAR	QPLARHFDP	WGQGTLLTVVSS
L35	TAIYYCAS	PLYPPKGPIVATTDY	WGQGTLLTVVSS
L52	TAVYYCAR	GCQHLVNYFDY	WGQGTLLTVVSS
L59	TAVYFCAR	DNGLPHNHFD	WGQGTQVTVSS
L69	TAVYYCAR	DKGLPYNHFD	WGQGTQVTVSS
L81	TALYYCAK	EGEQVG YFDWRTKLRF SFFDL	WGRGTLTVVSS
p7	TAVYYCAT	DGSR LSTSAFDFWSGNRPSSYIDV	WGKGTA VTVSS
p20	TAVYYCAR	RLIGGTFFPFRYSYVDV	WGTGTTVTVSS
p35	TAVYYCAR	DQGIRVAGGLDY	WGQGTLLTVVSS
L100	TAIYYCAK	GPLMRWFDD	WGQGTLLVAVSS
DO142-10	TAIYYCAR	SHCGSN CYGLFEH	WGQGTLLTVVSS

**Figure 1.** Amino acid sequences of the heavy chain CDR3 region and adjacent framework regions of anti-gp120 Fabs.

and is also abolished by a mutation in C5 (491 I/F), and is strongly impaired by a substitution in C3 (349 L/A).

A third epitope group of two Fabs (Fab L15 and L17) isolated by selection on ant-CD4bd mAb-captured gp120 is specific for the V2 loop. As shown in Figure 2, substitutions in or deletion of the V1/V2 loops abolished binding of Fab L15 to gp120. Fab L15 further competed with rodent anti-V2 mAbs SC258 (Figure 3(a)), CRA3, G3-4, G3-136, BAT-085 and 52-684 (data not shown) but not with anti-gp120 mAbs directed to other epitopes (Figure 3(a)). The binding pattern of Fab L17 is very similar to that observed for Fab L15 (not shown).

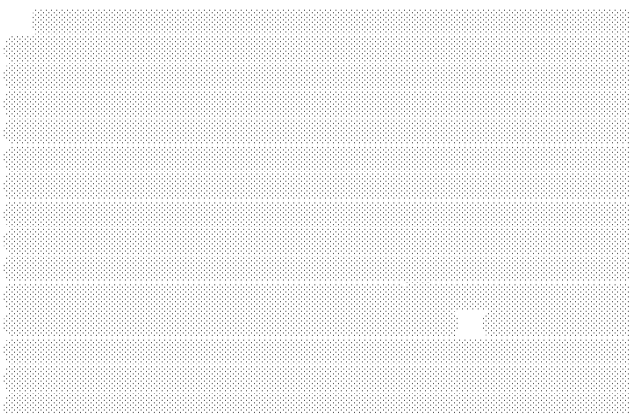
The fourth epitope group obtained by CD4bd-captured gp120 selection consists of the single antibody, Fab L25. The binding of this antibody to the panel of gp120 mutants demonstrated a sensitivity to substitutions in residues associated with the CD4 binding site and the V2 loop (Figure 2) as also observed for three previously described human Fabs (Ditzel *et al.*, 1995). Furthermore, Fab L25 competed for binding to gp120 with murine anti-V2 mAb SC258 (Figure 3(a)). This Fab is therefore directed to an epitope which we have termed the CD4bd/V2 loop-sensitive epitope.

### Multiple epitope masking

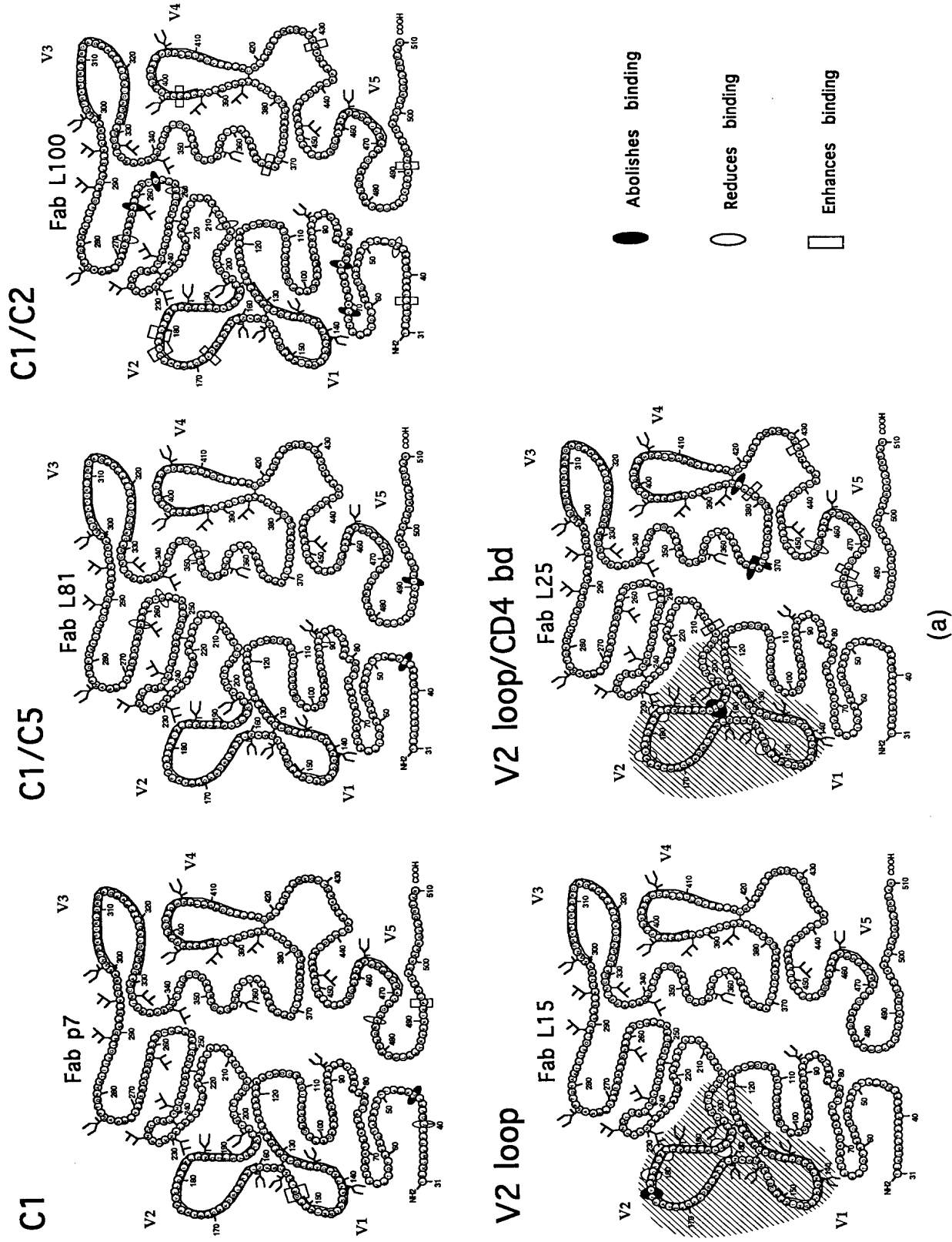
After selecting the pooled phage-display libraries for four rounds, two gp120 specific Fab fragments were isolated. Heavy chain sequence analysis identified one as anti-V2 loop Fab L17. The other represented a novel antibody, Fab L100 (Figure 1), which was found to bind to an epitope involving the C1 and C2 regions (Figure 2). Substitutions 69 W/L and 76 P/Y abolish the binding of Fab L100 implying that the antibody binds to a part of the C1 region distinct from that recognized by the

masking antibody, Fab p7. Substitutions 252 R/W, 256 S/Y, 262 N/T and 267 E/L abolish or strongly impair the binding of Fab L100, indicating direct or indirect involvement of the C2 region in the epitope recognized.

### Selection on peptides



Two neutralizing Fab fragments were selected. The first anti-V3 antibody, Fab loop 2, obtained by panning against the cyclic V3 loop peptide has been described (Barbas *et al.*, 1993). Fab loop 2 recognizes gp120 from HIV-1 strains MN and SF2 but not LAI. The second antibody, Fab DO142-10, was selected by panning against the RP142 peptide. The same Fab was also retrieved by panning against recombinant gp120 MN. To probe antigen specificity, Fab DO142-10 was tested for binding against a number of V3 loop peptides, recombinant gp120 proteins and unrelated antigens. As shown in Figure 4(a), Fab DO142-10 bound equally well to the RP142 peptide and gp120 MN, which were both used as selecting antigens. Lower binding affinities were observed with the PND peptide, a JR-CSF V3 loop fusion protein and a recombinant gp120 from primary isolate W61D. Fab DO142-10 did not bind appreciably to recombinant gp120 LAI or to a panel of unrelated antigens which included BSA, the Fc fragment of IgG and ovalbumin. Fab DO142-10



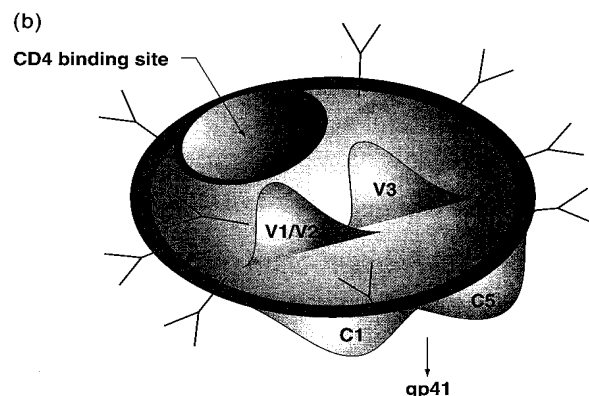


Figure 2(b)

**Figure 2.** (a) Relative binding of human recombinant Fabs to selected HIV-1 LAI gp120 (HXBc2) mutants. The primary sequence of gp120 is shown as a disulphide map. The position of putative N-linked carbohydrate attachment sites are indicated schematically. Only mutations abolishing binding (closed ovals; binding ratio  $<0.2$ ), decreasing binding (open ovals; binding ratio  $<0.5$ ) or enhancing binding (open rectangles; binding ratio  $>2.0$ ) of Fabs are indicated by superposition of oval or rectangle symbols on the primary sequence. The hatched region including V1 and V2 loops in the bottom two maps indicates strong impairment of Fab binding upon deletion of these loops. Mutations at positions that did not affect the binding of the Fabs shown are not indicated. Fab binding to the gp120 mutant panel was repeated three times with similar results. The following mutants were included in the panel tested: 36V/L, 40Y/D, 45W/S, 69W/L, 76P/Y, 80N/R, 88N/P, 102E/L, 103Q/F, 106E/A, 113D/A, 113D/R, 117K/W, 120/121VK/LE, 125L/G,  $\Delta$ 119-205 (V2), 152/153GE/SM, 168K/L, 176/177 FY/AT, 179/180LD/DL, 191/193YSL/GSS, 207K/W, 252R/W, 256S/Y, 257T/R, 262N/T, 166A/E, 267E/L, 169E/L, 281A/V, 298R/G, 313P/S, 314G/W, 356N/I, 368D/R, 368D/T, 370E/R, 370E/Q, 380G/F, 381E/P, 384Y/E, 386N/Q, 392N/E + 397N/E, 395W/S, 406N/G, 420I/R, 421K/L, 427W/V, 427W/S, 429K/L, 430V/S, 432K/A, 433K/A, 435Y/H, 435Y/S, 438P/R, 456R/K, 457D/A, 457D/R, 463N/D, 470P/L, 470P/G, 475M/S, 477D/V, 485K/V, 491I/F, 493P/K, 495G/K, 500/501 KA/KG. (b) A schematic model for gp120 structure. The diagram was adapted from Burton & Montefiori (1997), Sodroski *et al.* (1996), and Poignard *et al.* (1996). Extensive glycosylation is schematically indicated by Y-shaped protrusions. The C1 and C5 regions are accessible on monomeric gp120 but buried on native gp120 on the viral surface, probably by interaction with the transmembrane envelope glycoprotein gp41. gp120-gp41 heterodimers are present in the form of oligomers, probably trimers, on the cell or virion surface. The V1/V2, V3 regions and the CD4 binding site are accessible to antibody on monomeric gp120. These antigenic sites are less accessible, however, on gp120 in oligomeric configuration on the envelope of T-cell line adapted HIV-1, and exposure of the V3 region and CD4 binding site in particular are highly restricted on the envelope of primary viruses.

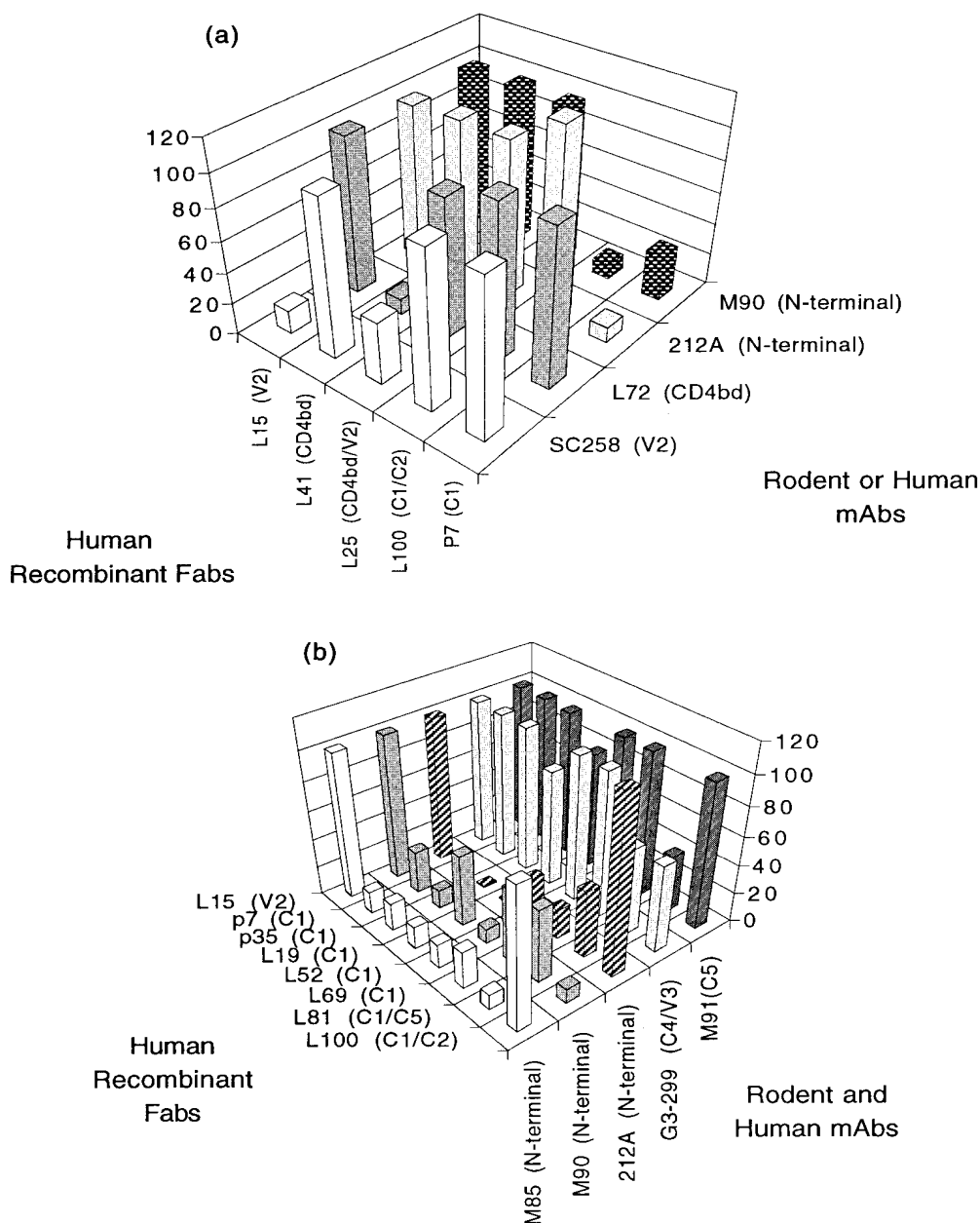
was also tested for binding to gp120 captured from a set of viral culture supernates which included HIV-1 AD6, JR-FL, AI-1, MN, SF2, 146, NYC-1, 120, and 437. Strong binding was observed to gp120 from HIV-1 MN and JR-FL, lesser binding to SF2 gp120 and no binding to the other gp120s (Figure 4(b)).

With respect to the V1 loop of gp120, we panned selected libraries against four linear 26-mer peptides corresponding to the sequences of the V1 region of gp120 from LAI and three primary isolates (case B, RA and VS). Serum titers of all eight library donors to the peptides were weak ( $<1:50$ ), and no V1-specific clones were isolated from the human libraries or from a library prepared from bone marrow of a chimpanzee infected with HIV-1 LAI.

### Further characterization of the N-terminal (C1) reactive Fab fragments

The selection experiments yielded Fabs to more than one epitope involving the C1 region. These Fabs were not retrieved by selection on gp120 directly coated to the solid phase. We decided to investigate these Fabs in more detail. The Fabs were cross-competed with a panel of rodent and human mAbs which included mAb M85 directed against a linear epitope in the extreme N-terminal region of gp120, mAbs M90 and 212A directed to different conformational epitopes in the N-terminal region, mAb M91 directed to a linear epitope in the C5 C-terminal region, and mAb G3-299 directed to the C4/V3 region (Figure 3(b)). The Fabs isolated by selection on soluble CD4-captured gp120 (Fab p7, p20 and p35) and mapped to a C1 region epitope competed with mAbs M85, M90 and 212A, but not mAbs M91 and G3-299. Fabs L52, L69, L34 and L35 retrieved by CD4bd antibody-captured gp120 showed a similar competition pattern as the p7 group. Fab L19 exhibited a broadly similar pattern but showed some inhibition of mAbs M91 and G3-299 and inhibited mAb M90 less efficiently. Binding of Fab L81, which was mapped to a C1/C5 region epitope, was inhibited by N-terminal reactive antibodies and mAb M91 directed against a C-terminal gp120 epitope. Fab L100 directed against the C1/C2 region did inhibit mAb M90 efficiently but not mAbs M85, 212A and M91. Some inhibition of the anti-C4 antibody G3-299 was also observed. The antibody inhibition studies are therefore consistent with the mutant mapping studies in identifying Fabs recognizing three distinct epitopes all involving the N terminus of gp120.

To elucidate why the extensive panel of C1 region reactive Fabs was not retrieved by selection with directly immobilized gp120, the Fabs were tested for binding to gp120 either coated to microtiter wells or captured by a sheep antibody (D7324) to the extreme C terminus of gp120. Interestingly, the C1 region reactive Fab fragments p7, p20, p35 and L19 did not bind to gp120 coated directly on the microtiter well (Figure 5(a)) (Fab p20

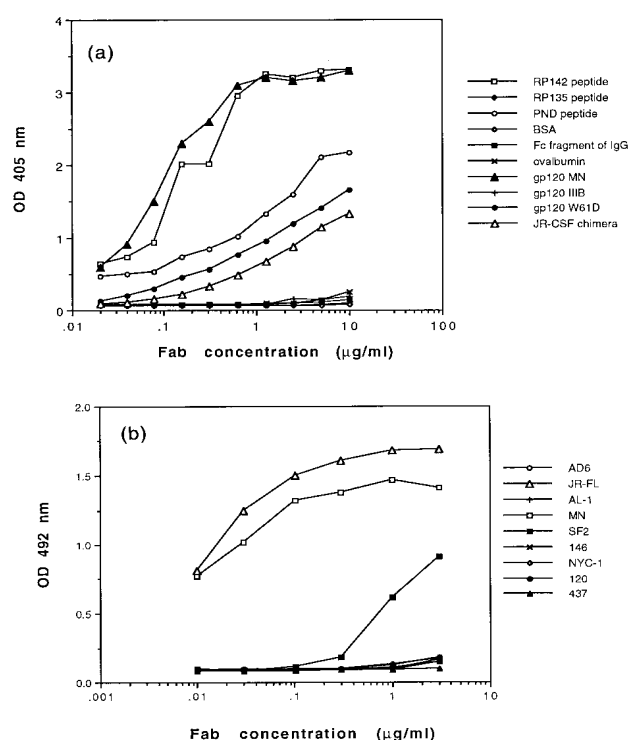


**Figure 3.** Competition between Fabs (a) directed to a diverse set of epitopes and (b) directed to overlapping N-terminal epitopes, with panels of rodent or human anti-gp120 mAbs. The epitope specificity of the human or rodent antibodies is specified between parenthesis. Bound rodent or human mAb (in a concentration giving 75% maximum binding) to gp120 LAI competed with the human Fab fragments at a concentration 100 times that giving 75% maximum binding in previous titration experiments was detected with AP-labeled anti-mouse IgG, anti-rat or anti-human IgG Fc antibody. Binding is expressed in %, with the  $A_{405}$  of uncompleted antibody set as 100%.

and p35 not shown). Other Fabs mapped to C1, such as Fab L69, in contrast, bound to gp120 in both assay formats, although the binding to captured gp120 was considerably stronger (Figure 5 (a and b)). CD4bd and V2 loop Fabs bound similarly to gp120 in both formats. This indicates that gp120 directly immobilized on to a microtiter well coats in an orientation which occludes part of the N-terminal region of the molecule.

We further examined whether Fab p7, p20 and p35 had higher affinity for captured gp120-CD4 complexes as compared to uncomplexed gp120. The Fabs bound efficiently in both cases. However, a small but significant increase in binding was seen to the CD4-complexed gp120, especially for Fabs p20 and p35 (data not shown).

To determine if the anti-C1 region Fabs recognized linear epitopes on gp120, gp120 was de-



**Figure 4.** Binding of the anti-V3 region Fab DO142-10 to: a, a panel of V3 peptides, recombinant gp120 proteins and unrelated antigens; b, gp120 captured from a panel of HIV-1 culture supernates using a polyclonal sheep antibody to the C terminus of gp120.

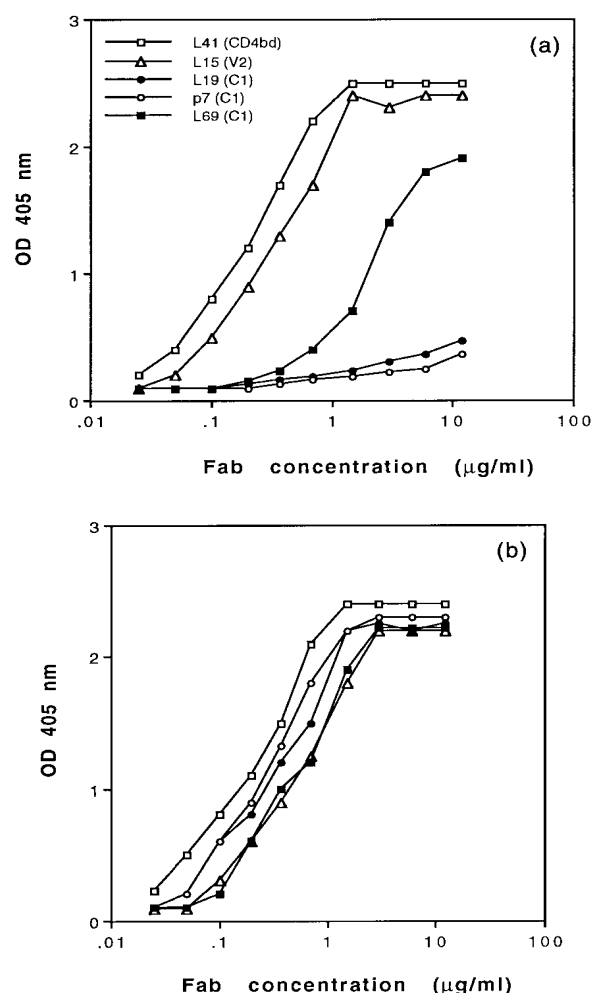
natured and captured by either anti-V3 mAb D47 or polyclonal antibody D7324. As shown in Figure 6, the binding of Fab p7 was reduced by denaturation but not completely abrogated. Similar observations were found for the other anti-C1 Fabs. As a control, antibody IIB-V3-13, recognizing a linear epitope in the V3 region, was run in parallel and was found to bind approximately equivalently to both native and denatured gp120 (data not shown) as reported (Moore *et al.*, 1994a).

### Fab affinity for recombinant gp120

The binding of a panel of selected Fabs to recombinant HIV-1 gp120 LAI and MN was measured by surface plasmon resonance using the BIAcore as shown in Table 1. Fabs p7 and L19 exhibited very weak binding to sensor chip-immobilized HIV-1 LAI gp120 and binding kinetics were therefore measured with an HIV-1 LAI gp140 oligomer. Affinities for all the studied Fabs were in the range of  $3 \times 10^7$  to  $4 \times 10^9$  M<sup>-1</sup>.

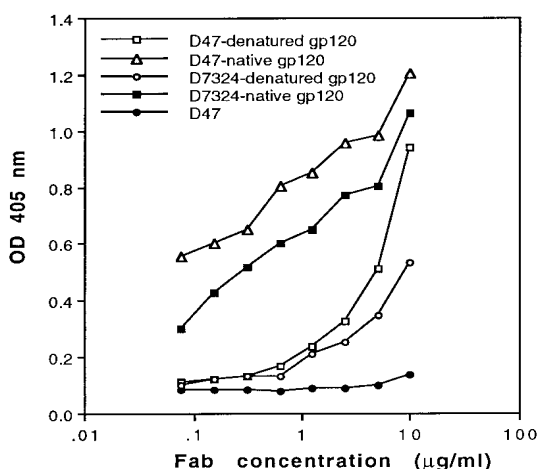
### Virus neutralization

Purified Fabs from each of the clones in the panel were initially examined for neutralizing ability in infectivity assays employing the MN and LAI



**Figure 5.** Binding of recombinant Fabs to: a, recombinant gp120 LAI directly coated on microtiter wells; or b, gp120 LAI captured by a polyclonal sheep antibody to the extreme C-terminus of gp120.

strains of HIV-1. Neutralization was determined as the ability of the Fab fragments to inhibit infection, as measured by a plaque reduction assay using MT-2 cells and syncytium formation using CEM-SS cells. In both assays none of the Fab fragments mapped to epitopes involving C1, including Fab L100, were neutralizing (data not shown). Anti-V2 loop Fab L15 also exhibited no neutralization. The CD4bd/V2 loop-sensitive Fab L25 showed only weak neutralization (data not shown). In contrast, in the plaque reduction assay, the syncytium inhibition assay and in an envelope-complementation neutralization assay, Fab DO142-10 demonstrated potent neutralization of HIV-1 MN with 50% neutralization titers at 0.2, 7 and 2 μg/ml, respectively. Weaker neutralization was observed against HIV-1 LAI, with 50% neutralization titers at 6, 8 and 8 μg/ml, respectively. The other V3 loop antibody, Fab loop 2, also potently neutralized HIV-1 MN with



**Figure 6.** Binding curves of Fab p7 (anti-gp120 C1 region) to native and denatured gp120 LAI captured on polyclonal anti-C terminal anti-gp120 antibody D7324 or anti-V3 loop mAb D47.

50% neutralization titers at 1, 5 and 2  $\mu\text{g/ml}$ , respectively. Fab loop 2 did not neutralize HIV-1 LAI.

## Discussion

Construction and selection of antibody phage display libraries offers an efficient route to obtain mAbs, including human antibodies (Burton & Barbas, 1994; Winter *et al.*, 1994). Considerable efforts have focussed on creating larger and more diverse naive or synthetic libraries where most reports describe the isolation of one or two antibodies to a given antigen. For some applications, including protein mapping, however, it may be essential to obtain antibodies to a range of epitopes. This may require more than simple selection of the library against the antigen of interest. Here we describe selection procedures leading to the isolation of an extended set of specificities to a single antigen. The antigen investigated is the HIV-1 surface glycoprotein gp120, which is a well characterized molecule with several advantages for this

study. These include the availability of many rodent and human mAbs to the molecule, synthetic peptides corresponding to linear epitopes of the molecule and an extensive set of mutant molecules. All of these resources assist in demonstrating the specificities of the antibodies retrieved from libraries. The approach described, however, should be generally applicable even in the absence of such resources.

Selection of HIV-1 immune libraries against recombinant gp120 yields overwhelmingly antibodies reactive with the CD4bd (Barbas *et al.*, 1993). One strategy to refocus selection was to capture gp120 on soluble CD4. This resulted in selection of Fabs reactive with an epitope overlapping the N-terminal (C1) region of gp120, a site which was shown by ELISA to be mostly occluded on gp120 coated directly onto plastic. These antibodies did bind to gp120 in the absence of soluble CD4, although some moderate enhancement of binding in the presence of the ligand was observed. This enhancement was less than that observed with the mAbs 17b and 48d, which are often described as binding to a "CD4-induced" epitope (Thali *et al.*, 1993). Mapping on gp120 mutants shows no evidence for the involvement of N-terminal residues in binding of the mAbs 17b or 48d.

Refocussed selection was also achieved using an anti-CD4bd mAb to capture gp120. The selected antibodies included several C1 region reactive Fabs as described above (p7 group of Fabs) but also another related epitope recognized by Fab L81 which involved residues from the N terminus (C1 region) but also residues from the C terminus (C5 region). A specificity for C5 was indicated by mutant binding analysis and competition with a murine mAb to the extreme C terminus. These results provide further evidence for the proximity of the C1 and C5 regions (Moore *et al.*, 1994b). Selection against CD4bd mAb-captured gp120 also yielded Fabs specific for the V2 loop and Fabs against a novel epitope which we have described as CD4bd/V2 loop-sensitive (Ditzel *et al.*, 1995). This epitope is not accessed by CD4-captured gp120 since it appears that the epitope is occluded on CD4 binding. Anti-CD4bd antibodies, in contrast,

**Table 1.** Kinetic constants and affinity constants for the binding of selected Fabs to gp120 measured by surface plasmon resonance

Fab	$k_{\text{on}}(\text{M}^{-1}\text{s}^{-1})$	$k_{\text{off}}(\text{s}^{-1})$	$K_{\text{a}}(\text{M}^{-1})$	$K_{\text{d}}(\text{M})$
b12	$1.1 \times 10^5$	$5.2 \times 10^{-4}$	$2.1 \times 10^8$	$4.7 \times 10^{-9}$
L17	$1.9 \times 10^4$	$1.9 \times 10^{-4}$	$1.0 \times 10^8$	$1.0 \times 10^{-8}$
L15	$1.1 \times 10^4$	$3.9 \times 10^{-4}$	$2.8 \times 10^7$	$3.5 \times 10^{-8}$
L19	$1.3 \times 10^5$	$3.6 \times 10^{-5}$	$3.6 \times 10^9$	$2.8 \times 10^{-10}$
L69	$5.5 \times 10^4$	$5.1 \times 10^{-4}$	$1.0 \times 10^8$	$1.0 \times 10^{-8}$
p7	$1.5 \times 10^5$	$1.0 \times 10^{-4}$	$1.5 \times 10^9$	$6.8 \times 10^{-10}$
DO142-10	$1.6 \times 10^4$	$1.8 \times 10^{-4}$	$8.9 \times 10^7$	$1.1 \times 10^{-8}$
Loop 2	$1.2 \times 10^4$	$2.3 \times 10^{-5}$	$5.2 \times 10^8$	$1.9 \times 10^{-9}$

Kinetic constants were measured with gp120 LAI for Fabs b12, L17, L15, and L69; with gp120 MN for Fabs DO142-10 and loop 2; and with a gp140 LAI oligomer for Fabs L19 and p7. The equilibrium association and dissociation constants were calculated from the experimentally determined kinetic constants with  $K_{\text{a}} = k_{\text{on}}/k_{\text{off}}$  and  $K_{\text{d}} = k_{\text{off}}/k_{\text{on}}$ .

enhance expression of this epitope. The observation that conformational changes occur upon antigen-ligand interaction, which may not be mimicked by antibodies to the ligand binding site, may be a feature of other antigen systems and should be borne in mind when developing selection strategies for phage-display libraries.

The strategy of refocused selection was further extended by masking the gp120 N terminus on the anti-CD4bd-captured gp120 by one of the C1 region reactive Fabs. This resulted in the selection of a Fab which recognizes a novel conformational epitope involving the C1 and C2 regions.

In only one instance did we select antibodies against the V3 loop by selection against gp120. This is despite the original designation of the V3 loop as the "principal neutralizing domain" of gp120. Several factors likely contribute to this paucity. First the human response to V3 during natural infection is probably less than that of mice immunized with recombinant gp120. Second we selected with gp120 of a single strain (mostly LAI) which almost certainly differed, particularly in V3, from the eliciting strain in the donor at the time of marrow collection for library construction. Third, because of variability the antibodies against V3 may be of lower affinity against "consensus" sequences than antibodies against conserved regions such as the CD4bd. Phage selection strongly favors clones of higher affinity (Barbas *et al.*, 1991) and so weakly cross-reactive anti-V3 clones may be lost during selection. The use of peptides can be useful in selecting antibodies reactive with largely continuous epitopes, as shown by the retrieval of two anti-V3 loop antibodies using V3 peptides.

The overwhelming majority of antibodies selected in this study showed poor or no neutralization of even T-cell line adapted strains of HIV-1 (primary isolates would probably be yet more difficult to neutralize (Burton *et al.*, 1994; Moore *et al.*, 1995)). Poor neutralization correlates with low reactivity with native multimeric gp120 on cell surfaces (Roben *et al.*, 1994; Sattentau & Moore, 1995), an extreme example being C1/C5 epitope antibodies which show no reactivity with cell surface envelope (Sattentau & Moore, 1995). However, the affinities of these antibodies for recombinant monomeric gp120 are shown by surface plasmon resonance studies to be high, suggesting they do result from antigen-driven processes. We suggest that this antigen is viral debris e.g. gp160 or shed gp120 generated during rapid viral turn-over (Ho *et al.*, 1995; Wei *et al.*, 1995) and not native virions. The antibody response to native virions may in fact be very limited.

## Materials and Methods

### Library construction and phage selection

Preparation of RNA from bone marrow lymphocytes and subsequent construction of IgG1  $\kappa/\lambda$  Fab libraries using the pComb3 M13 surface display system has been described (Barbas *et al.*, 1991; Burton *et al.*, 1991; Persson

*et al.*, 1991). For antibody selection, phage libraries generated from eight different HIV-1 seropositive donors were panned separately for the initial round and, after this, pooled together and panned additional rounds. The eight asymptomatic HIV-1 seropositive donors from whom bone marrow was aspirated for library construction have been described elsewhere (Ditzel *et al.*, 1994). Panning of the combinatorial libraries was carried out as described, with slight modifications (Burton *et al.*, 1991; Ditzel *et al.*, 1995). Baculovirus-expressed recombinant gp120, LAI strain (gp120 BRU, Intracel, Cambridge, MA) (0.1  $\mu\text{g}/\text{well}$ ) in PBS (phosphate-buffered saline (pH 7.4)) was captured by recombinant soluble CD4 (AIDS Research and Reference Reagent Program, Division of AIDS, NIH) (5  $\mu\text{g}/\text{ml}$ ) or a mouse anti-gp120 CD4bd mAb (mAb L72, kindly provided by Dr Hariharam, IDEC Pharmaceuticals Corporation, La Jolla, CA (Kang *et al.*, 1994)). In other panning experiments the following peptides or peptide-protein complexes were coated directly on ELISA wells: (1) a linear peptide corresponding to 24 residues of the HIV-1 MN V3 loop (RP142) (Repligen, Cambridge, MA) coupled to ovalbumin by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) conjugation (Pierce); (2) a linear peptide corresponding to 24 residues of the HIV-1 LAI V3 loop (RP135) (Repligen) coupled to ovalbumin; (3) a cyclic peptide  $\text{N} = \text{CH}-(\text{CH}_2)_3\text{CO}[\text{SISGPGRAFYTG}]\text{NH}_2\text{CO-Cys-NH}_2$  corresponding to the central most conserved part of the clade B V3 loop coupled to BSA; (4) a linear peptide corresponding to a "consensus" clade B V3 loop ("principal neutralizing domain" (PND) peptide); (5) four linear 26 amino acid residue peptides corresponding to the sequences of the V1 loop of gp120 from HIV-1 LAI and three HIV-1 primary isolates: case B, RA and VS (kindly provided by Seth Pincus).

### ELISA analysis

The human Fabs were purified from bacterial supernatants by column affinity chromatography using immobilized chicken anti-human Fab fragment. To assess specificity, supernatants were screened against gp120 and a panel of control antigens, which included BSA, ovalbumin, and the Fc fragment of human IgG (Sigma, St Louis, MO) by ELISA. Coating of ELISA wells was carried out as described (Ditzel *et al.*, 1995). Fabs were incubated with test antigen for two hours at 37°C, followed by washing ten times with PBS, 0.05% (v/v) Tween. Detection of bound Fabs was carried out with alkaline phosphatase (AP)-labeled goat anti-human IgG F(ab')<sub>2</sub> (Pierce, Rockford, IL) diluted 1:500 in PBS and developed with nitrophenol substrate (Sigma). Absorbance was read at 405 nm. To investigate if the epitopes recognized by the Fab fragments were conformational, gp120 was denatured and reduced by boiling for five minutes in PBS containing 1% (w/v) sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol (DTT) before ten-fold dilution into PBS containing 1% (v/v) NP40 to the concentration used (0.1  $\mu\text{g}/\text{well}$ ) (Moore & Ho, 1993). Native or denatured gp120 was then captured on a solid phase *via* the carboxy terminus using sheep polyclonal antibody D7324 (Aalto Bioreagents, Dublin, Ireland). A murine mAb IIIB-V3-13 (Laman *et al.*, 1992), which has been shown to react almost as well with denatured gp120 as with the native molecule, was used as a positive control. Binding of Fab DO142-10 to gp120 from a panel of different HIV-1 isolates was assessed by capture of gp120 from infected cell lysates as described elsewhere (Trkola *et al.*, 1995).

### Nucleic acid sequencing

Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (ABI, Foster City, CA) using a *Taq* fluorescent dideoxy terminator cycle sequencing kit (ABI). Sequencing primers were as reported (Ditzel *et al.*, 1994). The DNA sequences of the Fab heavy chains are accessible in Genbank under the following numbers (if two are given, the second number refers to the light chain sequence): DO142-10: U82961, U82962; L15: U82942; L17: U82943; L19: U82944; L25: U82945; L34: U82946; L35: U82947; L52: U82948; L59: U82949; L69: U82950; L81: U82951; L100: U82952; p7: U82767, U82768; p20: U82769, U82770; p35: U82771, U82772;

### CD4 and V1/V2 competition ELISAs

Recombinant gp120 was coated overnight at 4°C onto ELISA wells and blocked with 3% (w/v) BSA for one hour. Soluble CD4 in a tenfold dilution series ( $10^{-12}$  to  $10^{-6}$ M) or serial dilution of a HXB2d fusion protein (kindly provided by Abe Pinter) at a concentration of approximately 1 to 10 µg/ml was added together with Fab at a fixed concentration, previously determined to give 75% of maximum binding and incubated for two hours at 37°C. Following washing with PBS-Tween, bound human Fab was detected with AP-labeled goat-anti-human IgG F(ab')<sub>2</sub> and developed as described above. A HXB2d V3 fusion protein was used as a control antigen for the V1/V2 competition (Kayman *et al.*, 1994).

### Surface plasmon resonance to measure Fab binding affinities

The kinetics of Fab binding to recombinant LAI gp120 and a recombinant LAI gp140 preparation (Earl *et al.*, 1994) were determined by surface plasmon resonance using BIAcore (Parren *et al.*, 1996). Coupling of recombinant gp120 and gp140 to the sensor chip and subsequent binding of the Fab fragment to the immobilized antigens were performed as described (Binley *et al.*, 1996). The association and dissociation rate constants,  $k_{on}$  and  $k_{off}$  were determined as described (Karlsson *et al.*, 1991). Equilibrium association and dissociation constants were deduced from the rate constants.

### Epitope mapping by antibody cross-competition

Cross-competition experiments were performed between recombinant Fab fragments and a panel of murine and human mAbs. These included anti-C1 region mAbs: M85, M90, and M91 (kindly provided by Fulvia di Marzo Veronese) (di Marzo Veronese *et al.*, 1992); anti-V2 mAbs: 52-581-SC258 (SC258), 52-684-238 (52-684) (Moore *et al.*, 1993a) (kindly provided by Gerry Robey), CRA-3 (MRC AIDS Reagent Project, Potters Bar, Herts, UK), G3-4, G3-136, and BAT-085 (kindly provided by David Ho) (Fung *et al.*, 1992; Ho *et al.*, 1991; Sullivan *et al.*, 1993); N-terminal region mAb 212A (kindly provided by Jim Robinson); anti-C4 MAb G3-299 (kindly provided by David Ho) (Moore & Sodroski, 1996); and anti-V3 loops mAbs IIIB-V3-13 (AIDS Research and Reference Reagent Program, NIH) (Laman *et al.*, 1992) and D47 (kindly provided by Pat Earl) (Earl *et al.*, 1994).

Coating of gp120 onto microtiter wells was carried out as described (Burton *et al.*, 1991). Competing antibody at large excess (a concentration 100 times that giving 75% maximum binding in previous titration experiments) was incubated with the human Fab for two hours. Fol-

lowing washing, bound human Fab was detected, as described above. The assay was also reversed so that the human Fab was added at large excess (a concentration 100 times that giving 75% maximum binding in previous titration experiments). The murine antibody was detected with an AP-labeled goat-anti-mouse IgG (Pierce). Controls without competing antibody and with irrelevant antibody were included.

### Epitope mapping of Fabs by binding to gp120 mutants

For mapping of the binding site for the human Fabs, COS-1 cell expressed wild-type or mutant HIV-1 (HXBc2) envelope glycoproteins were captured onto solid phase using sheep-anti-gp120 antibody D7324, as described elsewhere (Moore *et al.*, 1993a,b).

### Neutralization assays

The human Fab fragments were assessed for their ability to reduce viral infectivity (HIV-1 MN or LAI) by a quantitative infectivity assay which enumerates multinucleate syncytia resulting from the fusion of infected CEM-SS cells with adjacent uninfected cells (Nara *et al.*, 1987), and a microplaque reduction assay using MT-2 cells as target cells (Hanson *et al.*, 1990). Virus stocks for both these assays were produced from chronically infected H9 cells. Selected Fabs were further tested for neutralization of HIV-1 infectivity by an envelope complementation assay assessing the ability of Fabs to inhibit a single round of viral infection (Helseth *et al.*, 1990). Viral stocks for this assay were generated by cotransfection of COS-1 cells by two plasmids, one expressing envelope glycoprotein, and the other expressing an envelope-deleted HIV-1 virus encoding chloramphenicol acetyl transferase as a reporter gene for infection of Jurkat cell targets. In all assays, controls without Fab and with the well-characterized neutralizing Fab b12 (Barbas *et al.*, 1992; Roben *et al.*, 1994) were run in parallel.

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obtained from the Medical Research Council, AIDS Reagent Project, National Institute for Biological Standards and Control, Potters Bar, UK.

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## EXHIBIT 4

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. : 09/854,568 Confirmation No.: 8438  
Applicant : SAMUEL BOGOCH  
Filed : May 15, 2001  
Title : METHODS AND COMPOSITIONS FOR STIMULATING THE  
IMMUNE SYSTEM  
Docket No. : 13793/46702  
Art Unit : 1644  
Examiner : David A. SAUNDERS  
Customer No. : 23838

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**SUPPLEMENTAL AMENDMENT UNDER 37 C.F.R. § 1.111(a)(2)**

Dear Sir:

This is a Supplemental Amendment to supplement the Interview Summary and Amendment and Response under 37 C.F.R. § 1.111(a)(2) filed April 4, 2007 in the above-captioned application. Applicant herein cancels previously withdrawn claim 6 without prejudice to facilitate further prosecution of claim 13 in co-pending U.S. Appln. Ser. No. 10/642,587. No extensions of time should be required for the instant Supplemental Amendment. However, if the Commissioner determines that an extension is required, Applicant hereby petitions for such extension and hereby authorizes the Commissioner to charge the payment of any required fees, including any extension fee, to Kenyon & Kenyon LLP Deposit Account No. **11-0600**.

Applicant respectfully submits the application is in condition for allowance.

**Amendments to the Claims** begin on page 2 of this paper.

**Remarks** begin on page 4 of this paper.

**Amendments to the Claims:**

This listing of claims will replace all prior versions, and listings, of claims in the application:

**Listing of Claims:**

1. (Previously presented) A method for killing glioma cancer cells in a subject wherein said glioma cancer cells express malignin, said method comprising administering to said subject an effective amount of a first dosage of a composition comprising malignin wherein said administration of said dosage stimulates the immune system of said subject to produce and release antimalignin antibody that binds and kills said glioma cancer cells.
2. (Previously presented) The method of claim 1 wherein the composition is administered as an approximately 1 mg dosage form.
3. (Original) The method of claim 1 further comprising administering a second dose of the composition ten days after administration of the first dosage.
4. (Original) The method of claim 3 further comprising administering a third dose of the composition ten days after administration of the second dosage.
- 5-6. (Canceled)
7. (Withdrawn) A device for removing cancer cells from the body of a subject, said device comprising cells having immunological specificity for malignin, recognin L or recognin M, wherein said device binds, absorbs or engulfs cancer cells which come into contact therewith.
8. (Withdrawn) The device of claim 7 wherein said device comprises a filter through which the cancer cells are passed.

9. (Withdrawn) The device of claim 7 wherein the device comprises a surface over which the cancer cells are passed.
10. (Withdrawn) The device of claim 7 wherein the device comprises a resin over which the cancer cells are passed.
11. (Withdrawn) The device of claim 7 wherein the cells are selected from the group consisting of T cells, B cells and phagocytes.
12. (Withdrawn) A process for ascertaining the presence of cells transformed to the malignant state in a subject
  - (1) obtaining a fluid or tissue specimen from the subject;
  - (2) quantifying the amounts of antimalignin antibody and/or immune cells having specificity for malignin, recognin L and/or recognin M in the fluid or tissue specimen;
  - (3) correlating the amounts to the presence of cells transformed to the malignant state.
13. (Withdrawn) An antimalignin antibody having a cytotoxic agent attached thereto.

**REMARKS**

Claims 1 to 4 are pending. Claims 5 and 6 are canceled. Claims 7 to 13 have been previously withdrawn in response to a Restriction Requirement. Applicant reserves the right to pursue canceled and withdrawn claims in a divisional application.

In this supplemental amendment, claim 6 has been canceled without prejudice to facilitate further prosecution of claim 13 in co-pending U.S. Appln. Ser. No. 10/642,587.

**CONCLUSION**

It is believed that the present claims are in condition for allowance and Applicant earnestly requests the same. An early and favorable action on the merits is earnestly solicited.

The Examiner is invited to contact the undersigned attorney if necessary to expedite allowance.

The Commissioner is authorized to charged any fees or overpayments associated with this application to Kenyon & Kenyon LLP **Deposit Account No. 11-0600**.

Respectfully submitted,

KENYON & KENYON LLP

/Richard W. Ward/  
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Dated: June 26, 2007

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## Electronic Acknowledgement Receipt

<b>EFS ID:</b>	1909560
<b>Application Number:</b>	09854568
<b>International Application Number:</b>	
<b>Confirmation Number:</b>	8438
<b>Title of Invention:</b>	Methods and compositions for stimulating the immune system
<b>First Named Inventor/Applicant Name:</b>	Samuel Bogoch
<b>Correspondence Address:</b>	KENYON & KENYON - Suite 700 1500 K Street, N.W. Washington DC 20005 US 2022204201 -
<b>Filer:</b>	Richard W. Ward
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<b>Attorney Docket Number:</b>	9425/46702
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Document Number	Document Description	File Name	File Size(Bytes)	Multi Part /.zip	Pages (if appl.)
1	Amendment - After Non-Final Rejection	SupplementalAmendment.pdf	88299	no	4

**Warnings:**

**Information:**

<b>Total Files Size (in bytes):</b>	88299
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**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.